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GOVERNMENT AND PRIVATE SECTOR
JOINT R&D PROJECTS

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**SCIENCE & TECHNOLOGY
JAPAN**

**GOVERNMENT AND PRIVATE SECTOR
JOINT R&D PROJECTS**

43066554 Tokyo SYMPOSIUM REPORT ON GOVERNMENT & PRIVATE SECTOR JOINT R&D PROJECTS in Japanese Jan 88

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List of Joint Projects for Biotech Research

43066554a Tokyo SYMPOSIUM REPORT ON GOVERNMENT & PRIVATE SECTOR JOINT R&D PROJECTS in Japanese Jan 88 pp 1-7

[Text] **Field I:** Development of Biotechnology as Foundation of Life Sciences; field chief: Akira Otani, National Institute of Health; number of themes: 7; number of tasks: 19; total funding for research: ¥320 million.

Research Theme 1: Development of technologies for isolating substances found in small amounts in the body (genes, enzymes, hormones, etc.); total funding for research: ¥73 million; leader: Satoshi Mizuno, National Institute of Health.

Research Tasks:

(1) Development of techniques for probing, separating, and purifying bioactive substances found in small quantities; Mitsuo Terao at the National Institute of Hygienic Sciences.

A. Development of techniques for efficient separation and purification of active factors for protein drugs produced by recombinant DNA.

B. Research on probing techniques regarding bioactive substances such as medicinal drugs (development of technology based on monoclonal antibodies).

C. Development of reagents for genetic engineering including restriction enzymes for intestinal bacteria.

D. Exploration, isolation, and culture of Eumycetes that produce bioactive substances.

E. Research on technologies for probing and isolating bioactive substances from marine microorganisms.

F. Determination of RNA structures of retroviruses and development of various kinds of monoclonal antibodies.

Research Organizations: Grelan Pharmaceutical Co., Ltd.; Sawai Pharmaceutical Co., Ltd.; Sankyo Co., Ltd.; Sumitomo Pharmaceutical Co.,

Ltd.; Takara Shuzo Co., Ltd.; Toyo Soda Manufacturing Co., Ltd.; National Institute of Hygienic Sciences.

(2) Study of bioactive substances governing cellular functions and research on their applications; Satoshi Mizuno at National Institute of Health.

A. Exploration of bioactive substances based on activation and selection of gene expression by chemical genetics.

B. Research on probing technologies regarding substances that inhibit expression of cancer gene function.

C. Research on detection, isolation, and application techniques of human cancer-caused bioactive proteins.

D. Development of probing and isolation technology regarding bioactive substances using automated high-sensitivity measuring method of cell function and research on its applications.

E. Development of high-performance carriers for high-speed liquid chromatography and isolation of substances found in small quantities in the body.

F. Exploration and detection of bioactive substances governing expression of tissue-specific genes and research on activation evaluation.

G. Detection of trace elements in the body based on culture of skeletal organs and development of an evaluation method of periodontal treatment.

H. Development of a new chemotherapy using modification of penetrability of animal and plant cell surface membranes and its utilization.

Research Organizations: Kyodo Shiryo Co., Ltd.; Kowa Co., Ltd.; Cosmo Kaihatsu (Development) Co., Ltd.; Toa Neryo Kogyo KK; Toyo Jozo Co., Ltd.; Mitsubishi Petrochemical Co., Ltd.; Meiji Seika Kaisha, Ltd.; The Lion, Co., Ltd.; National Institute of Health.

(3) Development of diagnostic technology based on nucleic acid hybridization and others; Akira Sugiura at the National Institute of Health.

Research Organizations: Eiken Chemical Co., Ltd.; Takara Shuzo Co., Ltd.; Nichirei Co., Ltd.; Fuso Pharmaceutical Industries, Ltd.; Yuki Gosei Kogyo Co., Ltd.; Wako Pure Chemical Industries, Ltd.; National Institute of Health; Research Laboratory of Public Health; Tokyo Metropolitan Government.

(4) Development of techniques for injecting nucleic acid into cells using high-frequency electric wave and micro-injector; Ichio Yanagi at the National Institute of Health.

Research Organizations: Olympus Optical Co., Ltd.; National Institute of Health.

(5) Isolation and purification of amyloid protein as a protein governing aging and diagnostic technology; Kazumi Iinuma at National Pediatrics Center.

Research Organizations: Sankyo Co., Ltd.; National Pediatrics Center, Tokyo Metropolitan University (Faculty of Science).

Research Theme 2: Development of vaccines by manipulating protein molecular structures; total funding for research: ¥37 million; research leader: Akira Otani, National Institute of Health.

Research Tasks:

(1) Development of multifunctional vaccine by molecular design; Akira Otani at the National Institute of Health.

Research Organizations: The ChemoSero Therapeutic Research Institute, The Kitasato Institute; Chiba Serum Institute; Daiichi Seiyaku Co., Ltd.; Teijin Limited; National Institute of Health; Institute for Neurosciences of Tokyo Metropolitan Government.

(2) Development of live vaccines; Takashi Kitamura at the National Institute of Health.

A. Development of vaccinia virus polyvalent live vaccine by recombinant DNA.

B. Development of mitigated picornavirus vaccine against poliovirus by introduction of gene defect mutation.

Research Organizations: Chiba Serum Institute; Nippon Zeon Co., Ltd.; Nippon Polio Research Institute; National Institute of Health; Tokyo University (Faculty of Medicine).

Research Theme 3: Development of host-vector systems using animal and plant cells; total funding for research: ¥36 million; research leader: Kunihiro Yoshiike, National Institute of Health.

Research Tasks:

(1) Development of host-vector systems using animal and plant cell cultures; Kunihiro Yoshiike at the National Institute of Health.

A. Development of host-vector systems using animal and plant cell cultures.

B. Improvement of the host-vector system in the actinomycetaceae streptomycetes--"kasugaensu" and research on gene expression.

Research Organizations: The Calpis Food Industry Co., Ltd.; Sanraku-Ocean Co., Ltd.; Shionogi Co., Ltd.; Meiji Milk Products Co., Ltd.; National Institute of Health; National Pediatrics Hospital; Cancer Institute Hospital; Keio University (Department of Medicine).

(2) Research on production of useful substances by transformation of medicinal plant cells; Notokichi Satake at the National Institute of Hygienic Sciences.

Research Organizations: Kanebo, Ltd.; Shiseido Co., Ltd.; Tsumura Juntendo, Inc.; The Lion Co., Ltd.; National Institute of Hygienic Sciences; Research Laboratory of Public Health of the Tokyo Metropolitan Government; Toyama Prefecture Pharmaceutical Research Institute.

(3) Safety evaluation of facilities and instruments such as physical confinement; Kazuya Yamanouchi at the Medical Science Institute.

Research Organizations: National Institute of Health; Tokyo University (Medical Science Institute) (IKAKEN).

Research Theme 4: Development of methods for testing and evaluating high sensitivity and high performance and safety of medical drugs; total funding for research: ¥54 million; research leader: Tadashi Takanaka, National Institute of Hygienic Sciences.

Research Tasks:

(1) Research on methods of verifying in vitro effectiveness and safety of chemical substances including medical drugs; Tadashi Takanaka at the National Institute of Hygienic Sciences.

A. Research on methods of verifying effectiveness and safety of medical drugs using culture systems.

B. Development of testing methods for safety evaluation of medicinal plant products by biotechnology.

C. Development of high-sensitivity simple testing methods by biotechnology.

Research Organizations: Upjohn Co., Ltd.; Otsuka Pharmaceutical Co., Ltd.; Sumitomo Chemical Co., Ltd.; Toyo Jozo Co., Ltd.; Fujisawa Pharmaceutical Co., Ltd.; Mitsui Petrochemical Industries, Ltd.; Meiji Seika Kaisha, Ltd.; National Institute of Hygienic Sciences; Food & Drug Safety Center; Tokyo University (Faculty of Pharmaceutical Sciences).

(2) Research on standardization of efficacy testing methods for drugs of the same kind and same efficacy produced by biotechnology; Akira Tanaka at the National Institute of Hygienic Sciences.

Research Organizations: Asahi Chemical Industry Co., Ltd.; Eisai Co., Ltd.; Kyowa Hakko Kogyo Co., Ltd.; Kowa Co., Ltd.; Sumitomo Seiyaku

(Pharmaceutical) Co., Ltd.; Toyo Soda Manufacturing Co., Ltd.; Toyobo Co., Ltd.; Mitsui Toatsu Chemicals, Inc.; Meiji Seika Kaisha, Ltd.; Mitsubishi Chemical Industries, Ltd.; The Green Cross; Mochida Pharmaceutical Co., Ltd.; Snow Brand Milk Products Co., Ltd.; National Institute of Hygienic Sciences.

(3) Development of technology for evaluating efficacy of antiviral drugs; Akira Otani at the National Institute of Health.

Research Organizations: Ajinomoto Co., Inc.; Seikagaku Kogyo Co., Ltd.; Meiji Seika Kaisha, Ltd.; Yamasa Shoyu Co., Ltd.; National Institute of Health; Shizuoka College of Pharmacy; Food and Drug Safety Center.

(4) Development of the method for standardization of potency of "cytokine" and safety evaluation; Shudo Yamazaki at the National Institute of Health.

Research Organizations: Asahi Chemical Industry Co., Ltd.; Otsuka Pharmaceutical Co., Ltd.; Suntory Limited; Mochida Pharmaceutical Co., Ltd.; National Institute of Health.

Research Theme 5: Development of technology for refinement and safety evaluation of newly developed foods; total funding for research: ¥33 million; research leader: Kunitoshi Yoshihira, National Institute of Hygienic Sciences.

Research Tasks:

(1) Research on utilization of biotechnology in foods and food additives and the method of evaluating chemical safety of products; Kunitoshi Yoshihira at the National Institute of Hygienic Sciences.

A. Development of the method for evaluating safety of products by tissue culture.

B. Research on identification of elements in natural foods and biotechnology-based foods.

Research Organizations: Ube Industries, Ltd.; Kirin Beer Brewery Co., Ltd.; San-Ei Kagaku Co., Ltd.; The Nikka Whiskey; National Institute of Hygienic Sciences; Food & Drug Safety Center; Tokyo University.

(2) Basic research on safety of biotechnology-based lactic bacteria; Kageaki Awaihara at the National Institute of Health.

Research Organizations: The Calpis Food Industry Co., Ltd.; Meiji Milk Products Co., Ltd.; Morinaga Milk Industry Co., Ltd.; Yakult Honsha Co., Ltd.; Snow Brand Milk Products Co., Ltd.; National Institute of Health.

Research Theme 6: Development of technology for isolating harmful substances by using microorganisms; total funding for research: ¥55 million; research leader: Yasuki Magara at the Institute of Public Health.

Research Tasks: Development of new waste disposal technology and safety evaluation support system; Yasuki Magara at the Institute of Public Health.

A. Development of raw sewage treatment technology and safety evaluation support system, using films.

B. Research on technology to treat hard-to-dissolve substances using biotechnology at the final waste disposal yard.

C. R&D of new water-quality evaluation technology using biosensors.

D. Research on biotechnology-based technology to remove harmful and pollution-causing substances in water leaking out of landfills.

Research Organizations: NGK Insulators, Ltd.; Institute of Public Health; Institute of Pollution Research; Japan Environmental Sanitation Center; Japan Environmental Improvement and Education Center; Waste Disposal Technology Development Center.

Research Theme 7: Development of experimental animals for life sciences research by developmental engineering; total funding for research: ¥17 million; research leader: Tateki Kikuchi, Seishin Shinkei (Mental and Nervous) Center.

Research Tasks:

(1) Development of experimental animals for medical research by developmental engineering; Tateki Kikuchi at Seishin Shinkei Center.

Research Organizations: Hassei Seishoku Seibutsu-Gaku Ken (Generation and Reproduction Biology Institute); Seishin Shinkei Center; Tokyo University (Institute of Medical Sciences); Kyushu University (Medical Department); National Institute of Basic Biology.

(2) Development of manipulation techniques of young embryos of experimental mammals and of development of experimental animals with disease using these techniques; Shigeo Honjo at the National Institute of Health.

Research Organizations: Eisai Co., Ltd.; Toyo Jozo Co., Ltd.; National Institute of Health.

Field II: Research on Evaluation, Improvement, and Development Technologies of Medical Materials as Foundation for Welfare Services; field chief: Tetsuzo Akutsu, National Cardiovascular Disease Center; number of themes: 5; number of tasks: 15; total funding for research: ¥280 million.

Research Theme 1: Research on the development and improvement technologies of high molecular film materials that selectively adsorb and fractionate harmful substances in the blood; total funding for research: ¥76 million; leader: Akira Yamamoto, National Cardiovascular Disease Center.

Research Tasks:

(1) Research on the development and improvement of the technology to selectively remove abnormal plasma proteins, aimed at prevention and treatment of aging and hardening of the arteries; Nobuhara Yokoyama at the National Cardiovascular Disease Center.

Research Organizations: Kanegafuchi Chemical Industry Co., Ltd.; National Cardiovascular Disease Center.

(2) Research on fractional specificities and blood compatibility of high molecular films or adsorbent agents to plasma protein; Akira Yamamoto at the National Cardiovascular Disease Center.

Research Organization: Kuraray Co., Ltd.; National Cardiovascular Disease Center.

(3) Development of methods to selectively remove disease-causing substances in the blood in hepatic insufficiency and renal insufficiency; Noboru Inoue at National Oji Hospital.

Research Organizations: Kuraray Co., Ltd.; National Oji Hospital.

(4) Research on development and improvement of the treatment of auto-disease by selective removal of specific auto-antibody; Norihisa Shibuya at the National Kawatana Hospital.

Research Organizations: Asahi Chemical Industry Co., Ltd.; National Kawatana Hospital.

(5) Basic research on application of high-performance affinity chromatography to medicine; Koichi Kojima at Food and Drug Safety Center.

Research Organization: Food and Drug Safety Center.

Research Theme 2: Research on technology for integrating high molecular materials with substances with pharmacological activity; total funding for research: ¥56 million; leader: Mitsuru Uchiyama at the National Institute of Hygienic Sciences.

Research Tasks:

(1) Research on the development and evaluation of high molecular materials for drugs; Yasushi Takeda at the National Institute of Hygienic Sciences.

A. Research on the speed of dissolution of macro molecules in biodegradable macromolecular drugs and release of the drug components.

B. Biological and pharmaceutical research on the design and evaluation technology of slow-releasing drugs.

Research Organizations: Eisai Co., Ltd.; Kowa Co., Ltd.; Sankyo Co., Ltd.; Shionogi & Co., Ltd.; Shimadu Seisakusho, Ltd.; Sumitomo Pharmaceutical Co., Ltd.; Daiichi Seiyaku Co., Ltd.; Taisho Pharmaceutical Co., Ltd.; Dainippon Pharmaceutical Co., Ltd.; Takeda Chemical Industries, Ltd.; Tanabe Seiyaku Co., Ltd.; Chugai Pharmaceutical Co., Ltd.; Fujisawa Pharmaceutical Co., Ltd.; Yamanouchi Pharmaceutical Co., Ltd.; National Institute of Hygienic Sciences.

(2) Development of macromolecular materials governing control of biological functions; Hiroo Iwata at the National Cardiovascular Disease Center.

A. Research on development and improvement technology of films with sensor function and control function.

B. Research on long-term extermination of in vivo destabilizing agents.

Research Organizations: Kyowa Hakko Kogyo Co., Ltd.; Toyo Soda Manufacturing Co., Ltd.; National Cardiovascular Disease Center; Kyoto University (Applied Polymer Research Center).

(3) Studies of simulation of human medicinal metabolism by combination of cytochrome P-450 molecular species and its fixation; Tadashi Takanaka at the National Institute of Hygienic Sciences.

Research Organizations: Sankyo Co., Ltd.; Shionogi & Co., Ltd.; Fujisawa Pharmaceutical Co., Ltd.; National Institute of Hygienic Sciences; Keio University (Department of Medicine).

Research Theme 3: Development of blood-compatible materials, and research on the improvement technology; total funding for research: ¥40 million; leader: Hisateru Takano at the National Cardiovascular Disease Center.

Research Tasks:

(1) Evaluation of development and improvement technology of macromolecular films with long-term effects against thrombus and for gas permeability; Hisateru Takano at the National Cardiovascular Disease Center.

Research Organizations: Toyobo Co., Ltd.; National Cardiovascular Disease Center.

(2) Research on evaluation of antithrombus effects of new materials; Takehisa Matsuda at National Cardiovascular Disease Center.

A. Development of silicone elastic bodies for chemotherapy, and evaluation of antithrombosis properties.

B. Study and evaluation of antithrombosis treatment by a catheter continuously measuring heart beat output.

Research Organizations: Kanegafuchi Chemical Industry Co., Ltd.; Terumo Corp.; National Cardiovascular Disease Center; National Pediatrics Hospital.

(3) Basic research on blood compatibility by extremely weak light-emitting phenomenon; Hiroyuki Nakasawa at the National Institute of Hygienic Sciences.

Research Organizations: Irika Kiki (Medical and Scientific Instruments) Co., Ltd.; Institute of Public Health; Food and Drug Safety Center.

Research Theme 4: Research on development and improvement of tissue-compatible (including durability) materials; total funding for research: ¥32 million; leader: Takehisa Matsuda at the National Cardiovascular Disease Center.

Research Tasks:

(1) Research on development and evaluation of tissue-compatible materials; Takehisa Matsuda at the National Cardiovascular Disease Center.

A. Development of surface reform technology of macromolecular materials and applications to medical instruments.

B. Biocompatibility and durability of hydroxyapatite.

C. Development of surface reform technology of metal and its applications to health and hygiene (growth of thin films by vapor deposition on artificial tooth and bone surfaces as wear-resistant hardening films).

Research Organizations: Ube Industries, Ltd.; Sumitomo Chemical Co., Ltd.; Sumitomo Cement Co., Ltd.; Nippon Tekken Co., Ltd.; Institute of Public Health; National Rehabilitation Center for the Disabled.

Research Theme 5: Research on testing methods as guidelines for effectiveness and safety evaluation of macromolecular materials; total funding for research: ¥42 million; leader: Mitsutada Nakamura at the National Institute of Hygienic Sciences.

Research Tasks:

(1) Research on safety evaluation method of polymer materials embedded in the body (development of in vivo and in vitro testing methods of tissue reactions in the body); Mitsutada Nakamura at the National Institute of Hygienic Sciences.

Research Organizations: National Institute of Hygienic Sciences; Medical Plastics Association; Ophthalmic Lenses Association; Artificial Kidneys Association; Science Association for Public Welfare.

(2) Research on tissue inhibition and pathogenicity of polymer materials and development of methods for their short-term assay; Hidehiko Izaka at the Food and Drug Safety Center.

Research Organization: Food and Drug Safety Center.

(3) Research on standardization of "limulus" tests to effect safety evaluation of high polymer films and their raw materials; Hironoshin Kawasaki at the National Institute of Hygienic Sciences.

Research Organizations: Seikagaku Kogyo Co., Ltd.; Daiichi Kagaku Yakuhin Co., Ltd.; Wako Pure Chemical Industries, Ltd.; High Polymer Separation Technology Association.

Field III: Elucidation of Defense Mechanisms in the Body as Foundation for Maintaining Health; field chief: Toru Tokunaga at the National Institute of Health; number of themes: 4; number of tasks: 14; total funding for research: ¥310 million.

Research Theme 1: Elucidation of the defense mechanisms in the body by immunity and phagocyte systems; total funding for research: ¥78 million; leader: Toru Tokunaga at the National Institute of Health.

Research Tasks:

(1) Research on the function and activation of macrophage; Yuzuru Akamatsu at the National Institute of Health.

- A. Function of macrophage and modification of its membrane structure.
- B. Research on intestinal flora and immunity.
- C. Research on analysis of functional control and physiological activity in immune system cells by polyunsaturated fatty acids.

Research Organizations: Idemitsu Petrochemical Co., Ltd.; Sankyo Co., Ltd.; Yakult Honsha Co., Ltd.; National Institute of Health; Keio University (Department of Medicine); Teikyo University (Department of Pharmacy).

(2) Research on cytokine and defense mechanism in the body; Toru Tokunaga at the National Institute of Health.

- A. Research on interleukin 2 inhibitor.
- B. Research on macrophage activator "lymphokine."
- C. Research on suppressor T cell factor.
- D. Research on cytokine and its network.

Research Organizations: Ajinomoto Co., Inc.; Ito Ham Provisions Co., Ltd.; Takeda Chemical Industries, Ltd.; Tsumura Juntendo, Inc.; Tokyo Immunity and Pharmacological Institute; Mitsui Pharmaceutical Co., Ltd.; National Institute of Health; Tokyo University (Department of Medicine).

(3) Research on how to reinforce the defense function of the body and how to control the function's abnormalities; Shigeo Honjo at the National Institute of Health.

A. Establishment of the technology for realizing immortality of B lymphocytes that produce human-specific antibodies, and its application.

B. Analysis and control of diseases caused by immune abnormalities.

C. Development of autoimmune disease models by anti-II type collagen.

Research Organizations: Ube Industries, Ltd.; Eisai Co., Ltd.; National Institute of Health; Tokyo Medical and Dental College (Department of Medicine); Tokyo University (Faculty of Pharmaceutical Sciences); Osaka University (Cell Engineering Center).

Research Theme 2: Elucidation of resistance to infection and defense mechanism of the body; total funding for research: ¥84 million; leader: Noboru Kobayashi at the National Pediatrics Hospital.

Research Tasks:

(1) Research on inflammation-causing substances in allergic conditions and their contagiousness; Yoji Iikura at the National Pediatrics Hospital.

A. Research on contagiousness of allergic conditions.

B. Isolation and identification of new inflammation-causing substances and their expression in large amounts of genetic engineering.

C. Research on the true fungus allergen that triggers allergy.

Research Organizations: Eisai Co., Ltd.; Mitsubishi Chemical Industries, Ltd.; Meiji Seika Kaisha, Ltd.; National Pediatrics Hospital; Food and Drug Safety Center.

(2) Analysis of pathogenic factors for defense from infection and research on the control of gene; Tae Takeda at National Pediatrics Hospital.

A. Development of a method to quickly diagnose enteric canal infection.

B. Genetic and biological research on defense mechanism in the body (the sensitivity between the 21st chromosome and down syndrome patients).

Research Organizations: The Shin-Etsu Chemical Co., Ltd.; Nissui Pharmaceutical Co., Ltd.; Hagiwara Health Research Institute; National Pediatrics Hospital.

(3) Immunological research on resistance to infection; Takao Kosaka at the National Pediatrics Hospital.

- A. Research on resistance to infection and liquid factors.
- B. Cellular and biological research on resistance to infection.
- C. Research on organ transplant aimed at preventing post-transplant infection.

Research Organizations: Kyowa Medics Co., Ltd.; Shimizu Pharmaceutical Co., Ltd.; Teijin Limited; Nisshin Flour Milling Co., Ltd.; Hagiwara Health Research Institute; Mitsubishi Chemical Industries, Ltd.; The Green Cross Corp.; National Pediatrics Hospital; Nihon University (Department of Medicine).

(4) Establishment of a new identification system using a specific antibody to blood cell reaction in the mechanism for defense against infection and its application; Junichi Hata at the National Pediatrics Hospital.

Research Organizations: Nippon Kagaku Co., Ltd.; National Pediatrics Hospital.

Research Theme 3: Elucidation of metabolism, detoxication and internal secretion functions, and the body's defense mechanisms; total funding for research: ¥76 million; leader: Kenji Yamaguchi at the National Institute of Nutrition.

Research Tasks:

(1) Elucidation of the organ-affinity mechanisms of elements found in small amounts in the body; Kenji Yamaguchi at the National Institute of Nutrition.

A. Elucidation of biological function and defense mechanism using an element identification mechanism.

B. Research on vascular specificity and metabolism mechanism of edible fats and oils.

C. Research on the effect of elements found in small amounts in the body on mammary gland tissue function and its metabolism.

D. Research on foreign matter metabolizing mechanism of white blood cells and its abnormalities.

E. Basic research on biosynthesis and cohesion metabolism functions of specific steroid hormone aimed at pathological analysis.

Research Organizations: Terumo Corp.; Toray Industries, Inc.; Nippon Bunko Co., Ltd.; Nippon Oils & Fats Co., Ltd.; Mitsubishi Petrochemical

Co., Ltd.; Morinaga Milk Industry Co., Ltd.; National Institute of Pollution Research; National Institute of Nutrition; Food and Drug Safety Center.

(2) Research on mutual reactions between nutritional elements having effects on bone metabolism and internal secretion functions (research and development of Ca drugs effective for bone metabolism in people who are pregnant, breast feeding babies, or at an advanced age); Yasukazu Ito at the National Institute of Nutrition.

Research Organization: Fuji Science, National Institute of Nutrition.

(3) Elucidation of the structure of "apolipo" protein and research on bio-defense mechanism; Hiroshige Itakura at the National Institute of Nutrition.

A. Detection of exogenous cholesterol and metabolism abnormalities by monoclonal antibody to apo B-48.

B. Elucidation of the role of bile acid in cholesterol metabolism structure.

C. Research on the relationship between apolipo synthesis system and cholesterol synthesis system.

Research Organizations: Sankyo Co., Ltd.; Tokyo Tanabe Co., Ltd.; Nippon Shoji Co., Ltd.; National Institute of Nutrition.

(4) Basic study of the reverse transfer structure of cholesterol heading from extremities to the liver and development of its activation technique; Akira Yamamoto at the National Cardiovascular Disease Center.

Research Organizations: Mitsubishi Chemical Industries, Ltd.; National Cardiovascular Disease Center.

Research Theme 4: Elucidation of the nervous and cerebral functions and of the body's defense mechanism; total funding for research: ¥64 million; leader: Yuji Miyamoto at the National Mental and Nervous Disease Center.

Research Tasks:

(1) Research on biodefense mechanism, and on specific protein in the brain and specific material in the nerves and their functions; Takeshi Tanaka at the National Mental and Nervous Disease Center.

(2) Recognition of brain cells in the biodefense reaction and amine metabolism.

A. Research on mutual recognition between the nerve cell and glia cells and on biodefense reaction.

B. Effect of stress on biodefense mechanism.

C. Research on causative substances of Parkinson's disease and on the body's defense mechanism to them.

Research Organizations: Asahi Glass Co., Ltd.; Asahi Breweries, Ltd.; Eisai Co., Ltd.; Kyowa Hakko Kogyo Co., Ltd.; Kirin Brewery Co., Ltd.; Suntory Limited; Sumitomo Pharmaceutical Co., Ltd.; Nichiray Co., Ltd.; Yoshitomi Pharmaceutical Co., Ltd.; National Mental and Nervous Disease Center; National Medical Center; National Uta Hospital; National Shizuoka Higashi Hospital; Keio University (Department of Medicine); Saitama Medical College; Juntendo University (Department of Medicine); Nagoya City University (Department of Medicine).

(3) Research on occurrence of mental dysfunctions; Makoto Hirano at the National Hizen Clinic.

Note: The name of the person (organization) appearing in the research column is the person responsible for the task.

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Biotechnology R&D To Lead to Improved Human Life

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[Text] Article by Katsutoshi Mitsuse, chief, Hygiene and Microorganism Department, National Institute of Hygienic Sciences: "Research on New Restriction Enzymes From Coliform Bacteria" [pp 8-9]

It is a well-known fact that the discovery of restriction enzymes has made possible recombinant DNA technology. At present, more than 100 kinds of restriction enzymes with different recognition segments have been reported. But there are problems with many of them such as the difficulty of handling restriction enzyme producing bacteria or the low enzyme yields. Consequently, most restriction enzymes are very expensive. Restriction enzymes produced from colon bacilli, on the other hand, have advantages: The colon bacilli are easy to handle; and there is a system established in which the enzymes can be produced in large quantities by cloning restriction enzyme genes into many copy plasmids, and thus they are very cheap. We have established a system in which restriction enzyme-producing bacteria can be easily detected from various pathogenic bacteria belonging to the intestinal bacteria, a relative of the colon bacilli. And we have discovered about 10 kinds of restriction enzymes helpful in recombinant DNA technology from typhoid, dysentery, and pathogenic colon bacilli. By introducing genes of only these enzymes into the colon bacillus K-12, it has become possible to safely produce restriction enzymes in large quantities in colon bacilli. We retrieved and purified restriction enzymes from salmonella, "erucinia," and cholera germs, the bacilli scarcely explored until now.

All the salmonella, erucinia, and cholera bacilli were derived from patients with the disease. The 120 salmonella strains were divided into about 20 serum types. Of about 100 erucinia strains surveyed, the Yersimia enterocolitica and Yersimia pseudotuberculosis each accounted for half. Most of the cholera bacilli were of the Er-Tor type. To detect the restriction enzyme producing bacilli, the method invented by Mise, et al. (Gene 44, p 165, 1986) was used. The purification of the restriction enzymes was based on Pirrotta and Bickle methods (Methods in Enzymology, 65, 1980).

Restriction enzyme-producing bacilli were observed in salmonellas, at high rates of above 20 percent. From the cutting patterns of λ DNA, these restriction enzymes are classified into at least more than four kinds. As of now, the existence of isoschizomers of KpmI, StyI, AvaII, and PvuII has been confirmed. Of these, the isoschizomer of KpmI produced by salmonella thompson (designated SthI) had a different cutting position from KpmI, at G/GTACC. The bacillus Asp718 was found to be an enzyme with the same cutting position and it has been placed on the market. When such factors as: it is impossible to obtain Asp718-producing bacilli and the Asp718 is a fairly hard-to-handle enzyme are taken into account, the high-yield SthI may be called a promising enzyme. Furthermore, when the following factors that GGTACC arrangement is included in the poly-linker-site of pUC18 plasmids and that the enzyme, differing from KpmI, produces single-chain DNA with a 5' terminal are taken into account, SthI enzyme will find wide application once placed on the market. At present experiments are underway to insert the gene of the enzyme alone into the colon bacillus K-12.

Ten strains of restriction enzyme-producing bacilli were discovered from erucinia but they were all isoschizomers of PstI (designated YenI). They were all discovered from the Yersimia enterocolitica serotype 08 alone. In the case of serotype 08, the existence of the enzyme was observed in 10 out of 12 strains. Thus, the detection of this enzyme will provide an important means for the detection and confirmation of the most pathogenic of all Yersimia enterocolitica, the serotype 08. The fact that YenI showed a high level of activation at a temperature as low as 15°C also draws attention. We have discovered four kinds of restriction enzyme-producing bacilli out of about 100 cholera germ strains. Judging from their cutting patterns λ DNA, we expect three of the four kinds are new types of restriction enzymes. At present we are undertaking experiments to introduce only restriction enzyme genes alone into colon bacilli.

Article by Kazuo Suzuki, chief of the Antibiotics and Living Organisms Office, Antibiotics Department, National Institute of Health: "Research on White Blood Cell Swarmer Factors Produced by Human Cancer Cells" [pp 10-11]

As cells responsible for cancer cell destruction in the defense mechanism of the living body, polymorphic nucleoleukocyte (PMN) has been reported to play a role, along with lymphocytes and macrophages, by discharging enzymes such as activated oxygen production and myeloperoxidase (MPO). It is assumed to be involved in the surveillance mechanism of cancer cell multiplication period. For PMN to reach the cancer tissue, it is believed that some kind of chemotaxis substance will have to be secreted from the multiplication segment of the cancer cell. Using the human macro-lung cell cancer Lu65C cell that shows PMN permeation and that has been established as the chemotaxis factor secreting cell clone as the model, we isolated and purified the PMN chemotaxis factor that the cell discharges by HPLC. We also obtained some knowledge about it. We designated the chemotaxis factor as Lu65C-derived-chemotaxis (hereafter LUCT). The objective of this research is to clarify the role of PMN in inhibiting cancer cell multiplication and the working mechanism of the chemotaxis, the PMN, in the surveillance mechanism, and at the same time to study its application as a useful marker in cancer diagnosis.

(Method) Culture method of Lu65C: Lu65C cells were first cultured in a 10 percent FBS added RPMI-1640 culture medium at a density of 1×10^6 cells/ml. When the cells became confluent, they were transferred to an 0.1 percent BSA added "Iskoph" synthetic medium for further culturing. Three days after the culture medium exchange there was observed a strong leukocyte chemotaxis activation in the culture solution. So, the culture medium solution on the third day was centrifuged at 3,000 rpm for 15 minutes at 4°C, and the supernatant was used as the sample for purification.

Swarmer activation measuring method: Swarmer activation was measured at each step of the purification process by the Boyden Chamber method characterized by good repeatability. Samples from each of the purification steps were placed in the lower chamber and in the upper chamber was placed a PMN suspended solution refined to 2×10^6 cells/ml, and the distances of the PMN that swarmed within a 3- μm pore area, 120 μm thick, in a 35-minute period under the conditions of 5 percent CO₂ and 37°C were observed by microscope. The swarm activation was evaluated by obtaining an average of values from the five fields of view. The bacteria swarmer peptide Met-Leu-Phe was used as the reference. The PMN was isolated from heparin-added human peripheral blood.

Purification method of LUCT: First, Lu65C culture fluid was subjected to a dialysis desalting to pure water; fractions that failed to be adsorbed by DEAE-Sepharose were adsorbed by CM-Sepharose to be eluted by 50 mM of a sodium phosphate buffer solution of pH 7.4 that contained 1.0 M of sodium chloride; and the result was a concentrated sample. Next, the obtained concentrated sample was again subjected to a dialysis for desalting using 500 mM of sodium phosphate buffer solution; and LUCT was isolated and purified by means of HPLC incorporating CM type ion-exchange chromatography Asahipak ES502C column (CM-HPLC), hydrophobic chromatography TSK gel Phenyl 5-PW column (Hydrophobic-HPLC), and negative-phase chromatography Cosmosil 5 TMS-300 column (RP-HPLC).

LUCT's uniformity and pI: The uniformity of LUCT was detected by letting TCA sediments ¹²⁵I labeled with lactoperoxidase through SDS-PAGE and autoradiography. pI was obtained by chromato-focusing using PBE118 Gel produced by Pharmacia Corp. and Pharmalyte pH 8-10.5 (column size 1.0 x 15 cm).

(Results) The swarmer activation of CM-HPLC was strongly observed in about 0.2 M-in-density elution fractions of sodium chloride. The activated fractions were used as samples for the next Hydrophobic-HPLC. In Hydrophobic-HPLC, the swarmer activation was observed most prominently in elution fractions containing about 1.0 M of ammonium sulfate, and those fractions were completely isolated from bovine serum albumin that had been added as a stabilizer. The activated fractions were used as samples for RP-HPLC. IN RP-HPLC, only two absorption (A_{210} nm) peaks (P1 and P2) were obtained. Swarmer activation was specifically observed strongly in P1 fraction but was scarcely observed in P2. About 1 μg of LUCT was obtained from 6 liters of the supernatant of Lu65C culture in the course of five purification processes. The yield was 1.8 percent, the relative activation

was 6.1×10^7 units/mg protein, and the purification degree was about 90,000 times. In the autoradiography of the P1 and P2 obtained in the RP-HPLC, P1 showed a single band in the vicinity of about 10 KD. The pI of LUCT was observed as a single peak in the vicinity of about 10.6 of pH.

(Observation) The molecular mass of LUCT was about 10 KD. A basic substance with pI of above 10.6, it was stable in both acid and alkaline solutions. It also was stable for more than 1 month at a temperature of -80°C. Judging from its physical properties and its molecular mass, we consider it to be a new swarmer protein. Studies are also being made on the sequence of amino acids on the N terminal side of the refined LUCT. Aiming at preparation of antibodies to the substance and at its production by genetic engineering, we are planning to have cloning of the gene.

Article by Tatsuo Miyamura, chief, Fourth Intestinal Virus Office, Intestinal Virus Department, National Institute of Health: "Research on Diagnosis Method of Infection of B-Type Hepatitis Virus by Nucleus Hybridization" [p 12]

With the practical use of the vaccine against B-type hepatitis (HB), the countermeasure policy against HB in Japan has entered a new phase. As for HB cases that occur following blood transfusions, medical accidents, and STD, proper countermeasures have been taken. Vaccines and HBIG are powerful preventive means. Also, a policy measure has been in place since 1986 for preventing HB virus-carrying mothers from infecting their new born babies.

At present, however, there are many carriers of HB virus in our country. The number is said to be 2 to 3 million. The group of HB virus carriers may become a new source of infection by itself, and the infections themselves are known to advance to chronic progressive diseases (hardening of the liver, liver cancer) at high rates. Consequently, countermeasures for carriers are acutely needed.

Since the infectivity titer of HB virus cannot be easily measured at present, detecting HBV-DNA from the carriers' blood or tissue is the only means available for quantitatively grasping the state of contagion. At the laboratory level, usually HBV-DNA is labeled using a radioisotope as a probe of nucleic acid hybridization. But, for hospitals and their laboratories which are being urgently asked to establish a policy for diagnosis and treatment of HB through detection of HBV-DNA, there are many problems needing solution such as cost, facility, and half-life. If it becomes possible to detect HBV-DNA using nonradioactive DNA probes without paying the price of reduced detection sensitivity, the technique will greatly contribute to the diagnosis and treatment of HB. The technique may also be applicable to papilloma virus and herpes virus infections.

Using HBV-DNA labeled with ^{32}P , we have detected and quantified HBV-DNA in the blood and liver tissue. And we have shown the availability of qualitatively measuring HBV-DNA at a sensitivity of 0.1 to 1.0 pg as well as the conditions for inhibiting nonspecific reactions.

This time, we are going to describe the current state of a detection system, a combination of a peroxidase labeled DNA probe ("Labezaim-POD) developed by our research cooperation group, Wako Pure Chemical Industries, Ltd., and a single-chain DNA probe manufactured by incorporating HBV-DNA into M13 phage, its tasks and its future prospective.

Article by Kazumi Iinuma, chief, Deformation Research Office, Department of Research Into Congenital Abnormalities, Center for Research and Treatment of Infants, National Pediatrics Hospital: "Research on Amyloid Protein in Alzheimer's Disease" [pp 13-14]

As a result of an analysis of a large number of familial samples, it was determined in the spring of 1987 that the locus of the gene for amyloid protein observed in Alzheimer's disease is in the 21st chromosome. The disease running in a family that shows an unusual autosome dominant heredity pattern. As a result of an analysis of DNA extracted from Alzheimer's patients, conducted simultaneously, it was reported that the specific regions for the 21st chromosome were redundant. A report on individuals who clinically show the characteristics of Down's syndrome but whose chromosomes show, under examination, a normal karyotype, was presented. Qualitative analysis of the SOD-1 enzyme activation on the 21st chromosome revealed that extremely small regions containing the locus of the gene in question were redundant. Long before the aforementioned research reports were presented, we had also noted that Down's syndrome patients had in them pathogenic factors of Alzheimer's disease which could provide clues to the mechanisms of aging. With aid from the HS Foundation for an aging-related research project, we were able to embark on such research.

Initially, we did not distinguish dementia due to advanced age from Alzheimer's disease and tried to analyze patients having these diseases as living materials for study. Thus, we started by analyzing proteins in the cerebrospinal fluid that perfuse the tissue closest to the central nervous system. But we finally came to the conclusion that as technology now stands, it is impossible to obtain sufficient amounts of protein necessary to carry out analysis on a steady basis. Since individuals who have been diagnosed as suffering from Alzheimer's disease are clinically distinguished from senile dementia patients of the so-called Alzheimer's type, we therefore decided to focus our research on cases diagnosed as Alzheimer's. From such a stand, we altered the research methodology aimed at distinguishing Alzheimer's-related proteins from the expression of protein in the gene. We embarked on the synthesis of partial DNA probes, selected from the sequence of bases for the ETS2 gene that is considered, based on available findings, to be at a farther locus than the SOD-1 gene.

On the other hand, we extracted the so-called amyloid protein from the insoluble contents of cerebral tissues obtained from Alzheimer's patients that remain intact even after SDS treatment. Using the protein as an antigen, we produced polyclonal antibodies in animal experiments. Our objective was to obtain a monoclonal antibody with a high specific antibody value from those polyclonal antibodies. I shall briefly explain the meaning of the analyses to be undertaken in the period that remains.

Article by Kuniaki Nerome, chief, Third Virus Office, Virus Rickettsia Division, National Institute of Health: "Molecular Structure of Vaccine: An Idea for Molecular Design" [pp 15-16]

Various vaccines have been developed by reparation of target genes for vaccines or manipulation of the structures of the concerned protein molecules. But progress has been very slow compared to expectations. To consider it from a different viewpoint, the whole process seems to typically show the frailty of advancing research and development based on a single technology, and I believe development of vaccines based on coordination of several technologies will help to ease the dangers.

Using the influenza vaccine as a model, we have been conducting research on the design alteration of the target gene and establishment of the evaluation system for its use as a vaccine, and on the development of proteins for vaccines and their molecular construction. Based on findings that we obtained in the course of joint research with two other organizations, I shall introduce the outlines of an idea and achievements obtained so far. Evaluation of the antigen-region on a single protein molecule is basic to developing a vaccine, so we adopted two strategies for determining the epitope. One is to forecast, using a monoclonal antibody, the useful epitope segment to be positioned in the area of an influenza virus hemagglutinin (HA), and a copy of the region was chemically synthesized. In other words, we added various carriers to various regions on protein molecules useful as vaccines to investigate their immune responses, to include the possibility of peptide vaccine. The second method was to clone the gene coding for HA protein, and we evaluated its immune response using vaccinia virus DNA. Therefore, we created special insertions made up of fragments of BamHI, SmaI, KpnI, SacI, and EcoRI so that HA regions of the vaccinia virus DNA can be utilized more easily. An advantage of this system is that the insertion of a foreign gene can be determined by observing the expression or nonexpression of HA activation of the vaccinia virus. To evaluate the system's plausibility, we inserted HA-DNA, cloned from A/Niigata/102/82 (H3N2) virus, into the SmaI segment and arrayed a promoter of 7.5 K at its tip. It was inserted, along with vaccinia virus, into a cell, and after going through homologous recombination, recombinant vaccinia virus was cloned. The resulting vaccinia containing the gene for influenza vaccine was injected into the tail vein of a mouse to examine in detail its immune response with time. The results revealed that the semi-live influenza vaccine produced in this experiment induced not only humoral immunity but also cellular immunity with cytolysis (CTL) activation as its guide. What this means is that if this system uses exogenous genes represented by the DNA that codes for hepatitis B surface antigen, polyvaccines can be produced easily.

Our third strategy was to arrange on a single protein molecule an epitope that can cope with a number of infections, and we built a model by altering the design of DNA that codes for two sub-HAs. Therefore, we examined the stereoscopic structures of H1 subtype and H3 subtype molecules, and searched for a site where the DNA coding for two proteins could be spliced. We cleaved the two DNAs at their RsaI positions and prepared frames by

adding to them BamI linkers, while taking care not to disrupt their stereoscopic molecular structures. In other words, we came up with a "hybrid" molecular design in which half of the protein molecule is a Hong Kong type (H3) and half is a HA (H1) of the Spain influenza virus. Results of the cloning and expression experiment revealed that it was acting on the chimera DNA, and we believe this finding may give direction to development of polyvalent vaccines in the future.

In the fourth stage of practical application, we plan to prepare an artificial film of B3-MDP, a kind of muramildipeptide with a glucan radical structure and to implant a protein for vaccine in the acyl group portion on its surface. Our recent studies on artificial film vaccine have revealed that the immune response of the B3-MDP-based artificial film vaccine not only increases the humoral antibody but also induces the CTL activation of lymphocytes in the blood, lungs or spleen. The expression of such a new function in immune response shows the way to future inert vaccines, and we think it may pave the way for development of vaccines not found in nature. Much will depend on whether we can develop the fundamental technology for embedding protein antigens for vaccines into artificial membranes.

This research was undertaken by the Third Virus Office of the National Institute of Health in cooperation with the ChemoSero Therapeutic Research Institute and Daiichi Seiyaku Co., Ltd.

Article by Hisayoshi Akagawa, chief of the Antibiotic Eumycetes Office, Antibiotics Department, National Institute of Health: "Improvement of Host-Vector System in Actinomyces and Expression of Genes" [pp 17-19]

Objective of Research: Actinomyces are an important group of bacteria that produce a variety of antibiotics. In the world of antibiotic-producing bacteria, the task is how to breed effective antibiotic-producing bacteria and how to enhance their yield by using recombinant DNA technology. Research on genetic engineering technology of *Streptomyces kasugaensis* with two kinds of plasmids, pSK1 and pSK2, has for a long time been at an advanced stage and it has officially been approved (August 1986) as a highly safe host-vector system in DNA experiments. Included in this study is basic research on how to make the system applicable to practical use and cloning and expression of the bacteria's biosynthetic gene lines since it produces the agricultural antibiotic substance Kasuga mycin. This time we worked to improve the vector and transformation systems.

Methods and Results:

I. Improvement on vector:

1. **Antibiotic resistance of *S. kasugaensis*:** The bacteria were tested against 19 kinds of chemical agents including erythromycin and kanamycin to see if they could live in an ISP No 2 agar culture medium plate containing antibiotics. The results showed that they display sensitivity to erythromycin (10 µg/ml), gentamicin (10 µg/ml), and kanamycin (50 µg/ml).

2. Applicability of existing actinomyces vectors pIJ61 (tsr: thiostreptone-resistant gene, aph: neomycin-resistant gene, pIJ702 (tsr, mel: melanin-producing gene), pIJ922 (tsr): In these vectors the host bacteria *S. kasugaensis* R6 strain (plasmid depletion strain) was transformed to examine the expression of marker genes. As the results show, the pIJ702 and pIJ922 transformed the R6 strain and showed resistance to thiostreptone. pIJ702, however, did not produce melanin and resulted in reduced copies.

3. Shuttle vector between *Bacillus coli*: Using the transformation system of *E. coli*, we ligated *E. coli* vectors pBR322 or pACYC184 to the pSK2-derived vector (tsr). These shuttle vectors transformed the R6 strain at a low frequency of 10/ μ gDNA or below.

4. Imparting of erythromycin-resistant gene (pIJ460) using mel (pIJ702), aph (Tn5) and mls: Using the transformation system of *S. lividans*, we conferred mel, aph, and mls genes to *S. kasugaensis* plasmid-derived vectors (tsr) and constructed vectors with an insertion lost activation region. Using them, we transformed *S. kasugaensis* and examined the expression of each of the marker genes and the stability of the vectors by examining the yields of vectors extracted from the cultured fungus bodies under nonselective pressure conditions. The results revealed that the vectors imparted with the mls gene raised the resistance of the host bacteria to erythromycin to more than 100 μ g/ml and were maintained stably. The vectors imparted with the aph gene elevated the host bacteria's resistance to kanamycin to more than 200 μ g/ml but for the retention of the vectors, it was necessary to add antibiotics to the culture medium.

On a protoplast regeneration medium added with tyrosine the host bacteria containing vectors imparted with the mel gene did not produce sufficient amounts of melanin pigments as to be discerned but the vectors were retained stably.

5. Imparting the promoter of mls to the structural gene of mel: By cleaving the mls' structural gene segment from a vector containing both the tsr gene and mls gene, we inserted the mel's structural gene directly behind the mls' promoter. The host bacteria containing the vector produced, on a protoplast regeneration medium added with tyrosine, melanin pigments, making it easy to distinguish them from nonproducing strains. Retention of the vector, however, was a bit unstable.

II. Improvements on the transformation system:

1. Regeneration of protoplasts: Using a culture medium that contained a shake culture medium, (0.4 percent glycerol, 0.3 percent polypeptone, 0.4 percent yeast extract, 0.1 percent glycine, 0.05 percent MgSO₄·7H₂O, 0.2 percent KH₂PO₄, 0.8 percent Na HPO 12H O [as published]), added with bovine serum albumin and succinic acid soda as an osmotic pressure stabilizer, we studied regeneration of protoplasts. The results revealed the following: Protoplasts were implanted in the main culture medium (1.8 percent agar); the surface was dried; and they were cultured at 27°C. In a week the

regeneration was complete, yielding a regeneration rate from 10 to 30 percent.

2. Transformation rate: Using R6 strain, pSK1-derived vector (tsr) and the protoplast regeneration system, we examined the fungus body culture time for the preparation of the transformation system and protoplasts. The results showed that the transformation rate was 5×10^4 - $5 \times 10^5/\mu\text{gDNA}$.

Consideration: The tsr, aph, and mls genes used as markers of the vector for *S. lividans* were expressed well in *S. kasugaensis*. The mel gene, on the other hand, obtained a high expression rate by the use of the mls gene's promoter. This suggests that among actinomycetes genes are contained those whose character expression takes different forms depending on the host bacteria, caused by their promoter activation expression. We have constructed vectors whose inserted activation loss is found only in BamHI, BglII, and SacI, by imparting those marker genes to two kinds of plasmids (pSK1, pSK2) of *S. kasugaensis*. The mel gene marker especially is helpful because it enables selecting colonies with recombinant DNA in protoplast regeneration medium. The use of the newly developed protoplast regeneration medium has enabled us to shorten the regeneration time to a week and to raise the transformation rate to more than $10^5/\mu\text{gDNA}$. The efficiency rate, however, has seen fluctuations from experiment to experiment, so further studies are needed. The transformation rate of the pSK2-derived shuttle vector extracted from *E. coli* after ligation to the *E. coli* vector pBR322 or pACY184 in *S. kasugaensis* was extremely low. This suggests that there is some restriction enzyme system in *S. kasugaensis*.

Article by Masao Yamada, chief, Gene and Chromosome Research Office, Hereditary Abnormalities Research Department, National Pediatrics Hospital's Research Center for the Treatment of Children: "Adenovirus Vectors" [pp 20-21]

With advances in genetic engineering technology and the growth of molecular biology, it has become possible to have molecular clones of genes, thus making it possible to introduce them into microorganisms and cells of mammals. In keeping with this development, various kinds of host-vector systems have been developed. While being extremely helpful in the analysis of base sequences that control gene expression, the techniques are also highly effective means for obtaining large quantities of proteins--products of the genes--for their direct analysis. The techniques are also expected to make it possible to produce in large quantities biological substances which either are found only in small amounts in nature or are hard to isolate, thus providing these products for use in the manufacture of reagents, medical drugs, and agricultural chemicals.

Bacteria represented by *E. coli* or systems with enzymes as their host like lower eukaryotes have advantages because they are easy to handle and can be cultured in large quantities cheaply. In fact, in the field of products mediated by human genes, several genes have been incorporated into the *E. coli* host to manufacture medical drugs, such as insulin. The gene-mediated products expressed in bacteria or eukaryotes, however, cannot expect to have the modification after transcription/translation that is available

with gene-mediated products of mammal cells. When such products are administered to humans as drugs, as a general rule it is desirable that they be products mediated by the same gene, and from the viewpoints of stability and biological activation, it is also desirable that they be properly modified. From such a viewpoint, development of a system that can express in large quantities molecular clones of genetic products is awaited.

Vectors using cells of higher mammals including man have been developed using virus-derived elements. These are broadly classified into two systems: the infection system that utilizes virus particles and the cell system, in which without letting virus particles form, plasmid DNA is introduced into cells by transfection and is maintained. The former infection system is today effective as a mass-production system. Among the former category are included SV40 and adenovirus systems, and among the latter is included the cell system involving multiplication using DHFR gene.

The author has been engaged in the development of a mass-production system using adenovirus as the vector. It has been confirmed that the produced protein has an enzyme activity and that it adds or secretes sugar chains. The expression level was about 1 percent of the total protein. (PRONAS 82:3567 (1985)) Focusing on this system's features and problems as well as the efforts for improvement, I shall compare my system with other systems and describe prospects for mass production systems in mammal culture cells.

Article by Kinzo Matsumoto, assistant, Toxic Pharmacology Class, Faculty of Pharmaceutical Sciences, Tokyo University: "Research on Working Mechanisms of Avermectins Using Cultured Nerve Cells" [pp 22-23]

Foreword: Avermectins (AVM) like Avermectin B_{1a} and Ivermectin (IVM) (a mixture of 80 percent IVM B_{1a} and 20 percent IVM B_{1b}) are drugs of the macrolide system and they have strong helminthic and insecticidal capabilities. Among the drugs with chemical structures resembling that of IVM is milbemycin D (MMD) belonging to the family of milbemycins. In terms of neurophysiology, AVM, it has been shown, open Cl⁻ channels related to the receptor for γ-aminobutyric acid (GABA), the inhibitory sex transmission substance, at the neuromuscular junction of crustaceans and thus diminish the membrane resistance of the synapsis rear membrane. Biochemically, on the other hand, AVM have been reported to perform the following functions of 1) to promote [³H]GABA efflux from the rat's cerebral synaptosome and 2) to modify the so-called GABA receptor complex which is made up of the GABA receptor, benzodiazepine receptor and Cl⁻ channel protein, allosterically at the synapse's rear membrane side. These findings relate to the pharmacological and toxicological functions of AVM, but there is little knowledge about the functions of AVM at the nerve cell level. In this study, with the objective of clarifying the working mechanisms of AVM on nerve cell membranes, we examined the functions of IVM and MMD using cultured spinal nerve cells with a great advantage that the same sample can be used for both electrophysiological and biochemical methods.

Method: In accordance with the Fischbach and Dichter (1974) method, cells treated by 0.25 percent trypsin and isolated from a spinal cord extracted from a 9 to 10 day-old chicken embryo were suspended in a culture fluid containing 5 percent chicken extract, 10 percent equine serum, and antibiotics and were incubated on a cover glass coated with collagen or in a culture dish. Multiplicative cells were inhibited using Fdu and uridine and were used in experiments 14 to 24 days later. Electrophysiological measurements were conducted based on the intracellular recording method using small glass electrodes and the whole cell clamp recording method using suction electrodes. For measuring GABA emission capability, cells were in advance incubated for 10 minutes in a solution containing [³H]GABA. After washing several times using 1 ml of nutritive liquid, the solution in the culture dish was replaced once every minute, and the level of [³H]GABA in the exchanged solution was measured.

Results and consideration: In the intracellular recording method, autonomous excitatory postsynaptic potential (EPSP) and inhibitory postsynaptic potential, as well as frequent generation of action potential triggered by EPSP, were observed. In these cells, the rest membrane potential was -49.1 ± 2.1 mV (mean \pm s.e.m; n=37) and the membrane resistance was 19.6 ± 1.9 M Ω (n=11). And in the majority of cells recorded, GABA led to changes in their membrane potential brought about by rises in conductance. In close to half of these cells, 1 μ m of IVM or MMD led to changes in their membrane potential and membrane conductance, similar to those for GABA. But different from GABA, there were observed cases in which repeated applications tended to diminish or weaken the functions of IVM and MMD. In cells which are relatively available for application again and again, when using K-acetic acid electrodes, the inversion potential was about -40 mV (n=2), and when using KCL electrodes, an inversion below 0 mV was not observed.

In the experiment using the whole cell clamp recording method, when the membrane potential was fixed at -40 mV and when the liquids inside and outside the cell were Na⁺-, K⁺-, and Ca⁺-free, GABA agonist, muscimol, and MMD generated Cl⁻ current. When Cl⁻ densities inside and outside the cell were the same, the inversion potentials for the two drugs were found in the vicinity of 0 mV. While the function of MMD was increased by 3×10^{-8} M diazepam, the same function was inhibited by 10 μ m picrotoxinin of GABA antagonists and 200 μ m bicuculline methobromide. On the other hand, when IVM was applied to 10-20 sec cells, its effect was irreversible. Although partially inhibited by picrotoxinin, IVM again showed its effect when the picrotoxinin was washed out.

As for [³H]GABA emission from cultured spinal nerve cells, no GABA discharge was observed, like the one observed in the 50 mM K⁺ stimulation, from any of IVM and MMD.

These results show the possibility that in cultured nerve cells of the spinal cord from chicken embryos, nerve endings already exist and the mechanism for incorporation and discharge of GABA is already in place, and that IVM and MMD instead of working through GABA discharge from the nerve ending, activate the Cl⁻ channel by working "on the region that differs

from the GABA recognition region but is related to the GABA receptor complex."

Article by Akira Tanaka, chief of the Biological and Chemical Department, National Institute of Hygienic Sciences, and Hisashi Kojima, a member of the HS Foundation: "An Attempt at Standardization of Measuring Methods of TPA Potency" [pp 24-25]

Foreword: The objective of this project is to standardize the methods of measuring the potency of the plasminogen activator (t-PA) of the tissue type and the pro-urokinase (pro-UK) which are thrombolysis agents of the same kind and same medical efficacy and to standardize the methods of displaying potency values. Medical drugs with the same efficacy, t-PA and UK are similar in many respects but the two also differ greatly in their nature. Consequently, imposing the same potency measuring method on t-PA and pro-UK could not necessarily be called appropriate. Therefore, we decided to conduct a study of the standard measuring method of potency for each of the two drugs.

Described below this time are results of an experiment conducted aimed at establishing the measuring method of potency of t-PA.

Method: Many measuring methods of plasminogen activator activity have been reported. Of these methods, we concentrated our research on the fibrin dissolution time measuring method (CLT method) widely used for measuring activity of drugs. In this method, we used the time needed for plasmin, an enzyme transformed from plasminogen by the act of t-PA, to dissolve a fibrin clot formed from fibrinogen by the action of thrombin.

Results: UK has until now been used as the standard thrombolysis agent, and we first directed our research to determining what product is best suited for use as the standard agent. There are many problems in using UK to measure the potency of t-PA, so we settled on using the standard product of t-PA.

Next, there arose problems as to the kinds of substrate, plasminogen and fibrinogen, and we conducted detailed examinations using types of fibrinogen derived from bovines and humans, as well as human-derived plasminogen with different molecular kinds. The results revealed that the dissolving time becomes larger depending on the density of the fibrinogen. However, there were differences depending on what sources the fibrinogen came from or on its specifications, and it has become apparent that in the CLT method, the kind of fibrinogen being used is an important factor. It has also been observed that plasminogen has a density range best suited to the CLT method. What kind of thrombin is used did not have any great effect on the CLT method.

Furthermore, we plan to report on the results of the measurements we conducted of the international standard t-PA and several kinds of t-PA by the CLT method.

Conclusions:

1. The fibrin dissolving time measuring method is handy, has high repeatability, and it was a method best suited to our objective.
2. As the standard product for measuring the potency of t-PA, the use of t-PA is considered appropriate.
3. It has become apparent that depending on the kind of substrate being used and its density, different measurement results are obtained.

By further conducting basic studies on the CLT method and by sorting out where problems lie, we plan to establish a standard method for measuring the potency of t-PA.

Article by Yoshinori Orikasa, chief researcher at the Drugs Research Laboratory of Meiji Seika Kaisha, Ltd.: "Study of the Model for Infection by Influenza Virus" [pp 26-27]

Objective: The objective is to establish a high-sensitivity experimental system that has high repeatability and matches well with clinical tests in order to develop an anti-influenza drug. In the experiment, we plan to infect ferrets. Besides studying the conventional judgmental items such as life or death and the amount of virus in the lungs, we plan to conduct research from the aspects of syndrome and physicochemical examinations to see if any useful data can be obtained for evaluating efficacy of drugs.

Materials and methods: We infected 42-week old male ferrets with 34 strains of influenza virus A/PR/8/ by either spraying or nasal drops. (The ferrets were obtained from the Hikari Plant of Takeda Chemical Industries, Ltd.) We then measured such things as the number alive, body weight, amount of feed intake, amount of water intake, amount of activity, temperature of the body surface, general symptoms, amount of protein in the mucus of the larynx, blood test value, antibody value, and the amount of virus in the liquids from the larynx and the nasal cavity to see if they could be used to judge the efficacy of drugs. As for the amount of activity, ferrets were videotaped at night using a high-sensitivity camera and the pictures were reproduced at high speed for analysis. As for body temperature, we measured the body surface temperatures of the animals to lessen burdens on them, using a radiation thermometer. We measured the amount of protein in the mucus of the larynx following the method by C.W. Porter, et al.

Results and consideration: There were observed differences in symptoms between the group of ferrets infected by spraying and the group of ferrets infected by nasal drops. Among the symptoms observed among the ferrets infected by spraying were reduced body weight, reduced feed and water intake, decreased activity, a drop in the body surface temperature, an increase in the number of respirations, and rhonchus. Against this, in the case of those infected by nasal drops the major symptoms observed were sneezing and nasal discharge. The differences in the symptoms are believed to have arisen from changes in the upper air-passage in the case of

infection by nasal drops and in the lungs in the case of infection by spraying. As for the "sneezing" and nasal discharge, there is no method to objectively quantify or measure the frequency of occurrence at this date, and further study is needed.

Conclusion: Infections by spraying and by nasal drops gave rise to different symptoms. In the case of infections by spraying the symptoms observed were reduced water intake, decreased activity, and an increase in respirations as well as fluctuations in measured values. By objectively quantifying these data, we believe these symptoms can be used as the basic data for evaluating drug efficacy. If the major symptoms of "sneezing" and nasal discharge observed in the case of infections by nasal drops could be quantified, we believe they could also be used for evaluating drug efficacy.

Article by Shudo Yamazaki, chief, Central Examination Department of Virus, National Institute of Health: "Research on Standardization of Potency of Cytokine and Its Safety Evaluation" [pp 28-29]

Objective: Development of new drugs based on biotechnology has been advancing remarkably in recent years, and of all this work, studies and experiments aimed at developing drugs based on various cytokines and their clinical applications have been undertaken at a rapid pace. To begin with, cytokines are natural bioactive substances secreted by cells in the body. However, as it has gradually become known that cytokines play a role as control factors of immune mechanisms which are the defense mechanisms of the body, there have been active efforts to develop drugs incorporating them in the hope of using them as preventive drugs against malignant tumors and various diseases that cause lowered immune functions. In Japan, development of drugs taking advantage of recombinant DNA technology in particular has been actively promoted. Several companies have already succeeded in developing interferon (IFN), interleukin 2 (IL-2), and cytokines like tumor necrosis factor (TNF) and these drugs are undergoing clinical tests. To scientifically evaluate the efficacy and safety of these drugs, it is necessary to establish a conjective evaluation standard for each of them. More than anything else, the problem of standardizing the method to evaluate the potency of each drug using the bioactivity specific to each is an indispensable basic research task to compare and analyze production lots and to compare the same drug from several companies.

International standard products have already been established for the α , β , and γ types of INF, thus establishing methods to standardize potency. However, there have yet to be established any internationally unified standards for cytokines like TNF, IL-1, and IL-2, so objective evaluation of the effectiveness of these drugs is lagging behind. In view of such a situation, this research was undertaken as a joint government-private enterprise project to develop methods to measure potency for cytokine drugs through bioassay and ELISA as well as to establish domestic standard products of such drugs.

Research results:

(1) The sensitivity of bioassay of TNF potency fluctuates greatly depending on the kind of cell used for bioassay and on the culture conditions. The L-M cell assay system, established after studying in detail the ranges of fluctuations caused by those factors, has proved to have excellent stability and repeatability as a bioassay system of TNF standard products.

(2) The provisional domestic standard product (γ H-TNF; J-PS5K01) adopted based on the above method has proved to be stable over a long period of time, and it can be used for standardization of potency of TNF drugs of both the recombinant and the natural type.

(3) We established a determination method of ELISA that is easier and faster than bioassay, and measured potency of TNF drugs produced by various companies. The results revealed that there is a high correlation between the potency values between it and bioassay.

(4) As for the establishment and standardization of bioassay systems for IL-1 and IL-2, conditions for stable maintenance of the sensitivity of the cells to be used in assay and for their freeze storage have not yet been established, and further detailed studies are needed.

Article by Kunitoshi Yoshihira, chief, Food Additives Department, National Institute of Hygienic Sciences; Emi Okuyama, research member in the field of fluids at the HS Foundation: "Comparison of Pigment Elements in Rubiaceae Plant Bodies That Produce Orange Pigments and in Their Cultured Cells" [pp 30-31]

With remarkable advances in biotechnology in recent years, production of useful substances in tank or plant levels by plant tissue culture and other methods has reached a practical level. But no study has yet been made on the safety of products manufactured using those advanced technologies. Production of substances using plant cell culture and other methods involves abnormal physiological states for plant cells such as dedifferentiation or transformation, and the metabolic products thus produced may be different from conventional products produced by the mother plants. Consequently, how to effect quality control, that is, safety control, of products obtained using these new technologies seems to be an urgent task. Our goal is to develop a chemical evaluation method in which we chemically compare products produced by means of tissue culture and others with their naturally produced counterparts for safety.

This time we conducted studies of rubiaceae plant bodies that produce orange-colored pigments and their cultured cells in order to examine products. In this project, we credit Yoshihiro Koseiki at Tokyo University for establishing tissue culture cell lines and Mikio Nakamura of San-Ei Kagaku Co., Ltd., for mass culture.

We obtained the materials, Rubia Akane Nakai and R. tinctorum L, from the pharmaceutical plant cultivation experimental farm in Tsukuba.

We obtained germ-free plant bodies from the seeds of these mother plants. We then incubated their tissue pieces in MS and LS basic culture mediums which are combinations of various plant hormones, observed how they changed, and selected strains characterized by high color tones. The results were that in the case of Rubia Akane Nakai, stable cultured cell lines were established in the 2,4-D (5×10^{-7} M) medium or the MS medium of 2,4-D (5×10^{-7} M) added with Kinetin (10^{-7} M). The obtained cultured cells of Akane were then put into a liquid suspension culture system. Our experiment to have a mass culture of the cells using a 10-liter jar fermentor revealed that systems using an LS medium added with NAA (5×10^{-7} M) produce favorable results.

In the case of *R. tinctorum* L., we obtained calluses of the plant using NAA (5×10^{-6} M) and Kinetin (10^{-7} M). We obtained favorable results in the mass culture of the cells in the NAA (5×10^{-7} M) medium and in the LS medium added with Kinetin (10^{-7} M), but we are still examining much better culture conditions.

We then obtained essences of the plant bodies thus obtained and their cultured cells by means of extraction. From the roots of Rubia Akane Nakai and *R. tinctorum* L., we obtained MeOH-extraction essences (Ra-1, Rt-1) and CHCl₃-extraction essences (Ra-2, Rt-2). From the cultured cells of both plants, we obtained essences (Ra C-1, Rt C-1) by means of MeOH extraction and essences (Ra C-2, Rt C-2) by means of CHCl₃. Considering the possibility that the extracted essences might contain glycosides, we subjected part of each to a hydrolysis treatment for a reextraction by AcOEt and examined the results.

To compare the extracts, we used an HPLC provided with a photodiode array. By comparing each peak's retention time and its UV/VIS spectrum with the standard samples, we identified the pigment elements contained. The column used for the HPLC was TSKgel ODS-120T, and the solvent had a gradient of MeOH-10 percent AcOHaq. As for the main peaks not observed in the standard products, we isolated the equivalent compounds and revealed their chemical structures by spectrum analyses.

Glycosides of 2-methyl-1,3,6-trihydroxyanthraquinone were mainly observed in the extract of Rubia Akane Nakai, the Ra-1, and the munjistin was also revealed. In the plant's cultured cell extract, the Ra C-1, glycosides were observed in extremely small peaks and munjistin showed main peaks. In the case of CHCl₃ extracts, 2-ethoxycarbonyl-1-hydroxy-anthraquinone and 1-hydroxy-2-methyl-anthraquinone showed main peaks in the plant extract, Ra-2, but similar peaks were not observed in the cultured cell extract, Ra C-2, and there the existence of pseudo-purpurin, munjistin, alizarin, and 2-methyl-anthraquinone was shown.

In the MeOH extracts of *R. tinctorum* L., the Rt-1, lucidin primeveroside and rubemythic acid showed main peaks, and in the cultured cell extract, Rt C-1, lucidin primeveroside and alizarin showed main peaks.

In the CHCl₃ extracts, besides alizarin and lucidin the existence of nordamnacanthal was observed in a relatively long retention time region in the plant extract, Rt-2, but in the cultured cell extract, C-2, peaks most likely representing pseudo-purpurin were characteristic besides those of alizarin.

As described above, we obtained calluses of rubiaceae plant bodies and cultured them in large quantities. Then, we compared pigment elements in the obtained cultured cells with those in the mother plant bodies. The results revealed that the main pigment elements in the two systems are derivatives of anthraquinone but that the two systems do not necessarily have the same derivatives.

We believe the use of HPLC provided with a photodiode array detector in the comparison has enabled us to conduct not only simple pattern analyses but also to make scientific comparisons at the material level.

At present, we are studying culture of *R. tinctorum* L. and are comparing the cultured cells with transformed cells.

Article by Masatake Toyoda, chief, Third Food Office, Food Department, National Institute of Hygienic Sciences: "On Identifying Similarities and Differences Between Natural Foods and Foods Manufactured Using Biotechnology" [pp 32-33]

In order to ensure the safety of foods produced by means of bioreactors and nutrient solutions for cultivation, in this research we conducted chemical analyses of such foods to determine their differences with natural foods regarding ingredients and to see if they contain harmful substances or not, as well as bioassays for comparison and examination.

1. Fruit juice drinks by bioreactor method

Because of the anticipated differences in the ingredients of foods depending on differences in the manufacturing method by bioreactor conversion, we examined two types of fruit juice drinks as to how their ingredients are affected by the fixation support, batch or continuous process.

Lactic acid bacteria fermentation grape juice was produced by letting a highly porous granular ceramic adsorb *L. casei* and by feeding it with processed grape juice. Among major ingredients that showed an increase under the new manufacturing method were free sterol and fatty acid, but the composition of amino acid also underwent a small change. The support was immobilized with bacteria at the rate of 10⁹/cm³. The sparkling apple juice was manufactured by letting alginic acid Ca beads take in and immobilize the cider yeast NDCC 391 and feeding the yeast with apple juice. Aromatic ingredients and alcohol were obtained efficiently at low temperatures of 10 to 15°C.

2. Comparing pigments in two types of vegetable, one cultivated on nutrient-containing solution and the other in soil

In studying the characteristics of newly developed crops, plants cultivated in a nutrient-containing solution are very handy. So, using leaf lettuce we compared amounts of chlorophyll and β -carotene contained in the vegetable. In the case of cultivations by nutrient-containing solutions, there were observed large differences in the amounts of chlorophyll content. To probe the causes for this, we attempted tissue cultures of the leaf lettuce.

3. Properties and distribution of Eumycetes in fermentation foods and tests for their mutation properties

In order to determine the possibility of Eumycetes producing harmful substances, we conducted studies of 152 strains of Eumycetes isolated and identified from various fermentation foods as to the possibilities of their producing harmful metabolites, but there were observed no problems. But mutagenicities of four types of "otane" carrots cultivated by different methods were negative.

4. Study of the analysis method based on enzyme immunity of T-2 toxin

We have developed a high sensitivity enzyme immune method for analysis of T-2 toxins that may contaminate newly developed foods. Capable of determining amounts in the 0.4 to 100 ppb, the method proved effective in the addition and recovery tests conducted using cashew nuts, popcorn, and corn.

From the foregoing, it can be said that provided there exist necessary technical conditions, production of biotechnology-based foods that are superior in both nutrition and flavor is available efficiently and at a high repeatability. There must be established methods for ensuring strict selections of the raw materials, sturdiness of the support for bacteria fixation, life of the embedded microorganisms, and precautions against infiltration of various germs. It is also considered absolutely necessary that newly developed foods are analyzed by gene map or isozyme for comparison with one another. Analyses by enzyme immunity methods will be frequently used in the future, but in some cases the specificity may not be perfect, thus demanding backup chemical analyses for confirmation.

Article by Hiroshi Ishida, chief, Water Treatment Plant Engineering Department, Kubota, Ltd.: "High-Load Denitrification Treatment of Raw Sewage Using Ultrafiltration Films" [pp 34-40]

1. Foreword:

With the development of biological nitrification denitrification method, raw sewage treatment technology has improved greatly in terms of BOD and the removal rate of total nitrogen. But since separation of microorganisms from the treated water is conducted by means of gravity sedimentation or centrifugal separation, the treatment capabilities of microorganisms cannot be said to be utilized to the fullest. A solid-liquid separation method by ultrafiltration films is one

of the processes that can solve the problem. The adoption of the method would not only enable realization of bioreactors from the diluted self-immobilizing and mixing microorganism system to be high-density selective microorganism system but increase by far the safety against pathogenic microorganisms, thus hopefully elevating the efficiency of the high-level treatment process for removing substances which are hard for microorganisms to break down.

2. Nitrification denitrification reaction of raw sewage

Raw sewage contains BOD of 8,000 to 14,000 mg/l and total nitrogen of 3,200 to 5,200 mg/l. Under conditions where oxygen is being supplied fully and there remains little BOD, ammonia is oxidized according to the formulas shown below:



Under oxygen-deficient conditions (below DO 0.5 mg/l) where hydrogen donors are plentiful, on the other hand, NO_2 and NO_3 are reduced to N_2 gas according to the following formulas:



Various methods of raw sewage treatment have been developed based on biological nitrification denitrification technology, but every process utilizes the reactions shown in (2) through (4).

An example is shown in Figures 1 and 2. In the former half of the cycle there is little DO even when the sewage is aerated and denitrification reactions come first. Next, when dumping raw sewage is suspended, there is a decrease in NH_4^+ and almost no increase in $\text{NO}_x\text{-N}$. This indicates that the nitrification reaction and denitrification reaction are progressing efficiently. In the latter half of the cycle, as the amounts of organic matter remaining in the sewage become smaller, the denitrification efficiency goes down and the amount of $\text{NO}_x\text{-N}$ increases gradually. When the nitrification reaction has run through its course and there remains almost no NH_4^+ , DO increases rapidly. Determining whether the DO has shown a rapid increase or not at the end of the cycle will make it possible to know whether the air supply for the process was adequate or not. By taking advantage of this, the amounts of air to be pumped can be automatically controlled.

3. Solid-liquid separation by ultrafiltration films

In treating raw sewage by biological reaction tanks or ultrafiltration films, it is important to understand various factors governing the speed of sewage flowing through the film (flux) and the maintenance of the long-term stability of flux. In order to show the factors governing flux, we

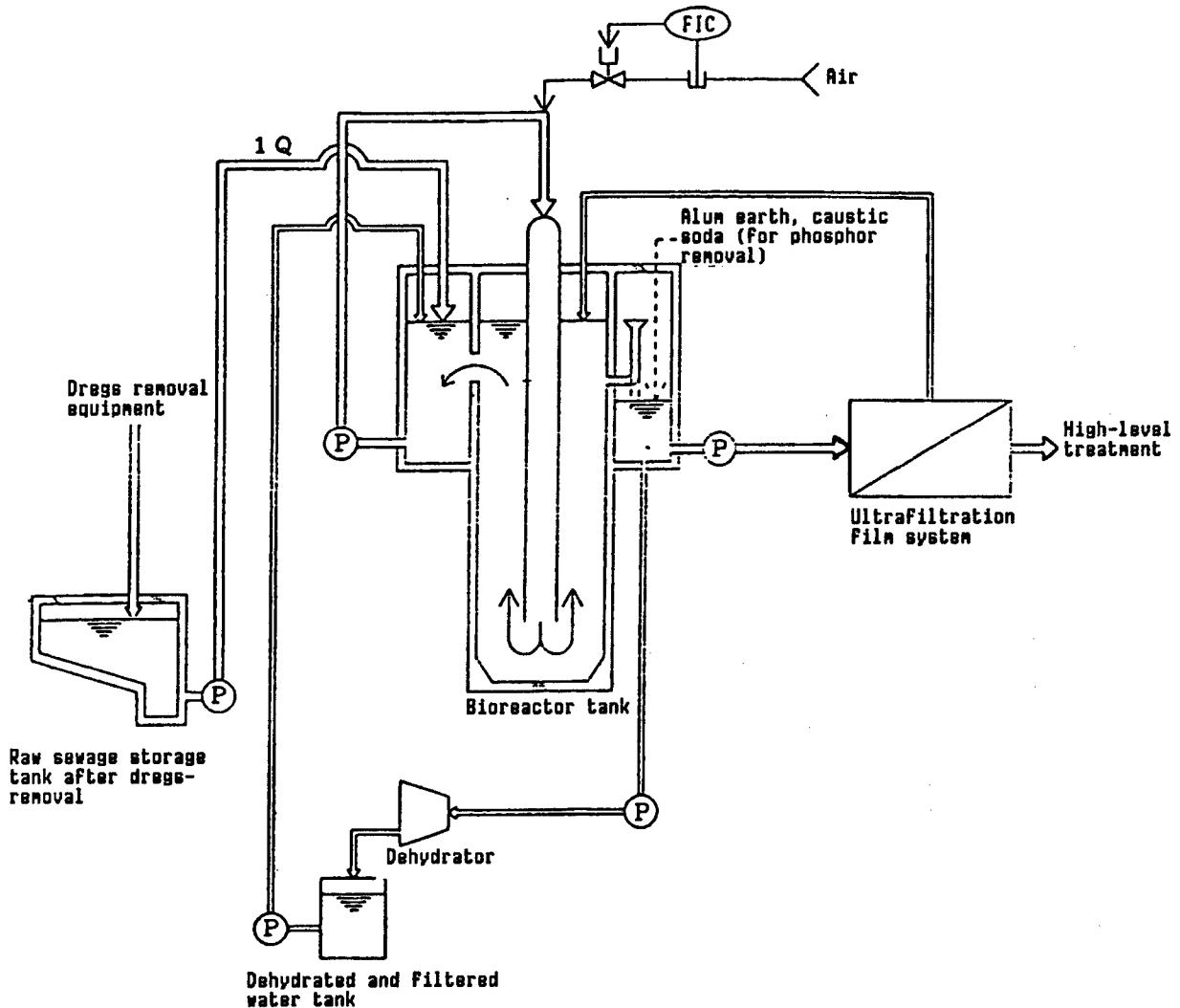


Figure 1. Demonstration Test Equipment Flow Sheet

conducted membrane separation tests on mixed liquids obtained after denitrification treatment of raw sewage, using a polyolefin tubular internal pressure type module with a graduated molecular weight of 20,000. The results are shown in Figures 3 and 4.

(1) Relationships between the operating pressure and flux

In the case of tap water, 20 times-diluted activated sludge-treated mixed solutions low in organic matter density, or membrane-separated liquids' coagulating mixed liquids, the flux increases in proportion to increases in the pressure. In the case of undiluted activated sludge-treated mixed liquids, the flux levels off when the pressure exceeds 3 kg/cm^2 . (These liquids are high in organic matter density.)

This is probably caused by the following reason: An increase in the flux leads to polarization of density and increased concentrations of soluble

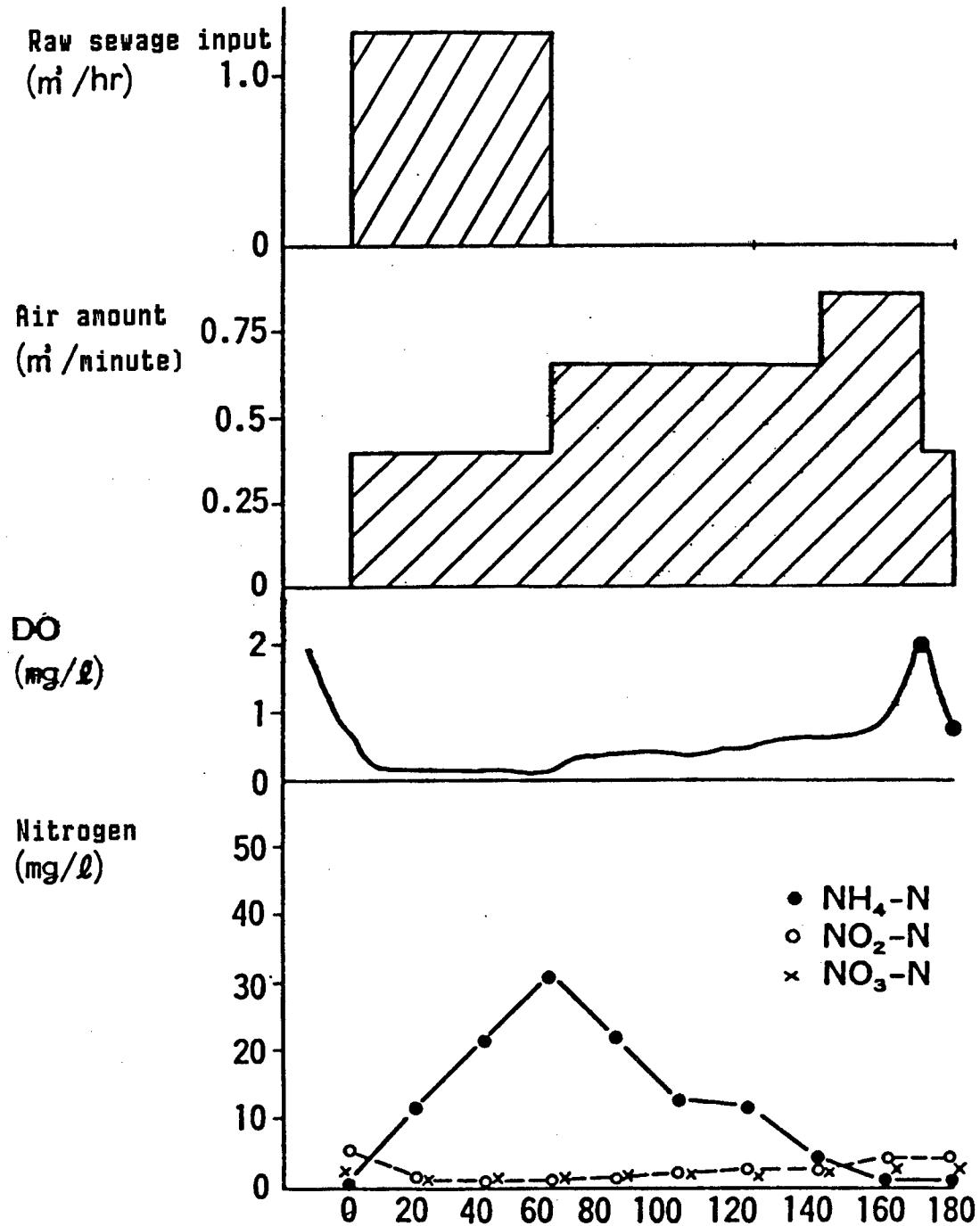


Figure 2. Changes in Bioreactor Tank With Time

organic substances on the film surface turn those substances into gels. This phenomenon is observed regardless of differences in film materials or graduated molecular weight. Even films that initially show a higher level of flux tend to show about the same values as the other films in their final flux, regardless of differences in film materials or graduated molecular weight.

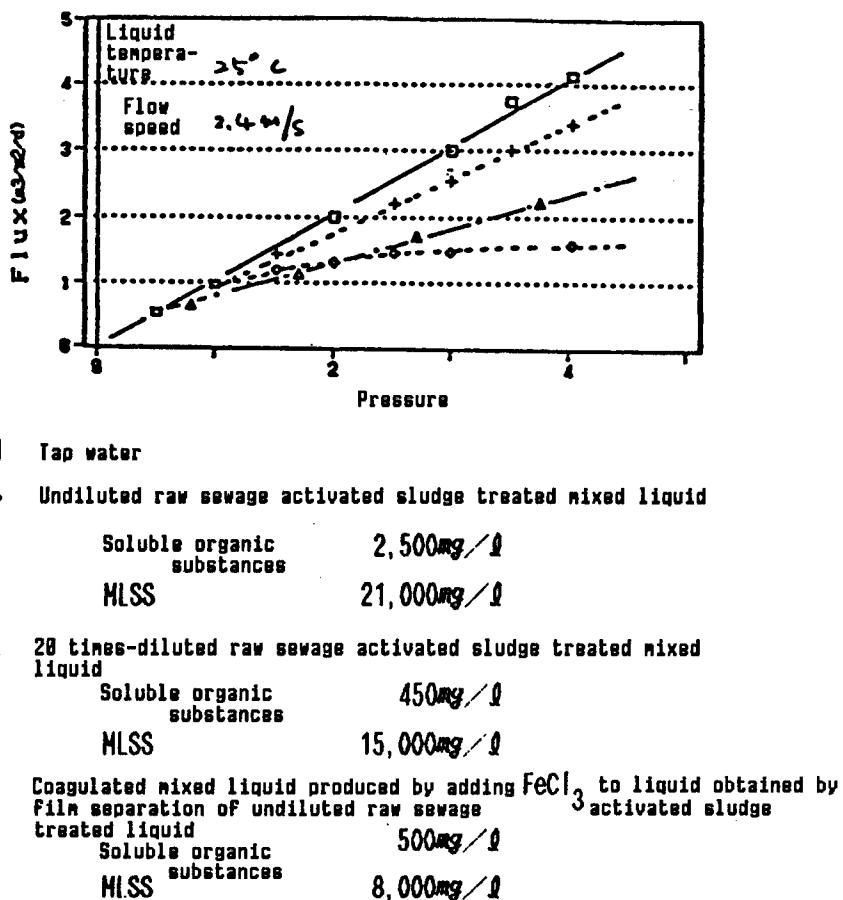


Figure 3. Effects of Pressure on Flux

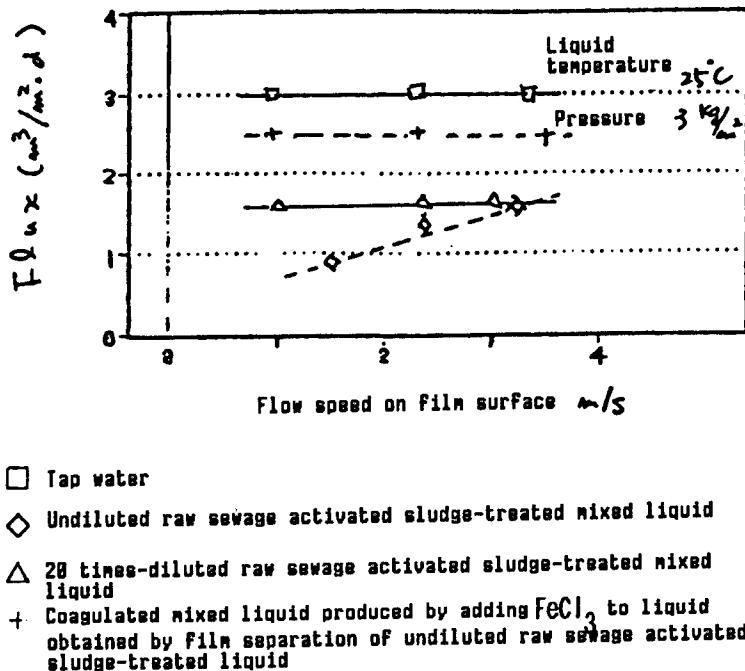


Figure 4. Effects of Flow-Speed on Film-Surface on Flux

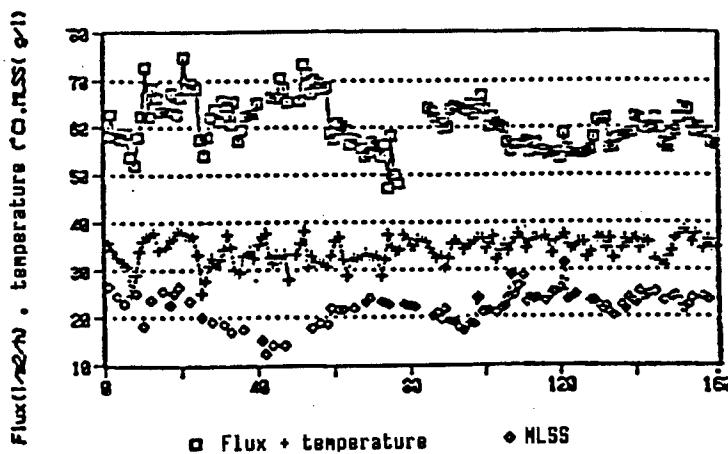


Figure 5. Endurance Test Results of Film
(Records after 2 years of use)

Table 1. Results of Demonstration Tests (capacity 10 k ℓ /day)

	Dregs-removed raw sewage (rough screening)	Ultrafiltration film permeation water
pH	8.2 - 8.4	7.0 - 7.2
BOD	mg/ ℓ 7757 - 10495	4.6 - 8.6
COD	mg/ ℓ 5052 - 5686	191 - 225
SS	mg/ ℓ 9583 - 12205	ND
Rough fibers	mg/ ℓ 3429 - 5383	ND
Total nitrogen	mg/ ℓ 3244 - 3890	32 - 40
PO ³⁻	mg/ ℓ 654 - 912	2.7 - 6.0
Cl ⁴⁻	mg/ ℓ 2425 - 2825	2199 - 2583
Chromaticity	-	600 - 730

Reaction conditions

Liquid temperatures--33-36°C
 MLSS--20521-21763 mg/ ℓ
 T-N/SS load--44-55 g/kg/SS/day
 BOD load--1.33-2.3 kg/m³/day

(2) Relationships between flow speed on the film surface and flux

In the case of tap water or 20 times-diluted activated sludge-treated mixed liquids low in soluble organic substance concentration, the flux does not increase even when the flow speed on the film surface is increased. In the case of undiluted activated sludge-treated mixed liquids, high in soluble organic substance concentration, however, the flux goes up as the flow speed increases.

(3) Durability tests of films

We conducted tests to see the durability of films using a demonstration experimental system with a sewage treatment capacity of 10 kl/day. The results are shown in Figure 5 and Table 1. The flux took different values according to the liquid hs. Even after the films had been in use for about 2 and 1/2 years, their flux, as shown in Figure 5, showed no increase or decrease, and was stable.

(4) Conclusion

The use of ultrafiltration films in solid-liquid separation has yielded, as shown in Table 1, further improvements in terms of BOD and the total nitrogen removal rate. Our research results on the factors governing flux have revealed that concentrations of soluble organic substances have great effects on flux. The continuous operation test lasting about 2 and 1/2 years has confirmed the system's practicality.

The gel layers formed as a result of the polarization in the concentration of the soluble organic substances are inferred to keep small holes from being clogged.

Article by Arihiro Hashimoto, assistant chief researcher, Developmental and Reproductive Biology Research Institute: "Experimental Manipulation of Primordial Germ Cells" [pp 41-42]

Primordial germ cells which have the role of transmitting genetic material to the next generation are expected to have, different from somatic cells, totipotency, and research on PGC has mainly been promoted using salientian Amphibia. In the case of mammals, because proper markers have yet to be discovered at the cell level and experimental methods have yet to be established, much remains unknown about their PGC including the cell lineage. If it becomes possible to experimentally manipulate PGC and insert the manipulated PGC again into the reproductive cell systems of individuals, new approaches to research could open up in the study of formation mechanisms of reproductive cells and in application fields such as medicine and agriculture. In this research we aim to develop basic technology for producing gene-introduced animals (new animals which can possibly be used for experiments) by introducing isolated PGC into exogenous genes and then inserting those PGC into the reproductive cell systems.

Appropriate markers for PGC in mammals have yet to be discovered. So, we pursued the behavior of PGC normally generated in a mouse by histochemically detecting alkaline phosphatase (ALP) activity.

Among the ICR line (closed colony) and C57BL/6 line we used no conspicuous differences caused by developmental stages or differences in ALP activity were observed. PGC appear for the first time in proximals of the allatonic bladder (back terminals of the embryo) of 7.5 to 8.5 day old embryos. In 9.5 to 10.5 day old embryos the PGC shift from the back mesenterium to the genital ridge, all the while increasing their numbers. In 11.5 to 12.5 day old embryos the PGC reached the genital ridge one after another and established themselves. In 12.5 day old embryos identification of males from females can be made from morphological features of their genital glad rudiments and it was found that contained in the genital glad rudiments at this stage of embryonic development were about 2,000 PGC cells. So, we decided to use 12.5 day old embryos as the starting materials.

In order to experimentally manipulate PGC, the following three basic techniques must be established. They are 1) the method of isolating PGC, 2) the method of culturing PGC, and 3) the method of introducing PGC into individual animals. As for items one and three, we believe the reconstituted ovary method developed by Motoko Noguchi, et al., at Shizuoka University may be effective, and we are now studying conditions for the experiment. An outline of the experiment is as follows: By taking advantage of the relatively weak adhesion of PGC to the culture dish, PGC are isolated from somatic cells; then, (engineered) PGC are again mixed with the somatic cells for a revolving culture, which produces aggregates of cells. Then, the aggregates are transplanted beneath the cysts of the ovary of adult mice whose ovaries were removed to trigger formation of organs. by crossing such host mice, PGC-mediated offspring can be obtained. We have yet to obtain any offspring, but we believe that if the optimal experimental conditions are found, the technology will have a high level of utility.

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Medical Materials R&D for Medical Treatment

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[Text] Article by Nobutaka Tani, chief researcher, Central Research Laboratory, Kanegafuchi Chemical Industry Co., Ltd.: "Adsorption of LDP by Dextran Cellulose Sulfate and Its Application to Treatment of High Blood Cholesterolemia" [pp 44-45]

A rise in blood plasma cholesterol density is a dangerous factor not only in arteriosclerosis but also ischemic heart disease in particular. These diseases are caused mainly by an increase in the cholesterol level brought about by blood plasma low-density lipoprotein (LDL). The density of high-density lipoprotein (HDL) is widely known to be correlated with crisis situations and progress of arteriosclerosis. The most distinguishing feature of these diseases is cholesterolemia running in the family. A sharp rise in LDL leads to the development of juvenile arteriosclerosis, and its homozygote in particular is accompanied by a great change to a morbid condition.

When the blood plasma LDL is lowered to levels below an epidemiologically observed threshold that causes arteriosclerosis, the chances of having ischemic heart disease decrease to levels where the contribution of LDL is nil. The stage where a drastic lowering of the LDL density can be expected to lead to decreased diseased arteries has been reached.

As for cases of hard-to-cure high lipemia centered on high cholesterolemia running in the family, on the other hand, it is known that lowering the LDL density in the blood by drugs, such as anti-lipemia agents, alone entails much difficulty. To cope with such a disease, development of a method is awaited that will enable us to directly and selectively remove LDL in the blood without lowering the HDL density.

Aiming at developing a system that selects and removes LDL with high efficiency by using an adsorbent which has special affinity with LDL and by letting the patient's blood plasma circulate through the adsorbent outside the body, we designed the adsorbent, evaluated it, and manufactured it. Then, we used the system in the treatment of cases of hard-to-cure high lipemia.

Development of Adsorbent

The affinity adsorbent is made up of a ligand specific to LDL and a support that is used to keep in fixation and maintain the ligand. In the selection of the ligand, several candidates were selected in the following criteria: 1) their toxicity is so low that even if the entire ligands should be desorbed, safety can still be maintained, 2) they are stable even after being subjected to rigorous treatments such as high-pressure steam sterilization. After screening tests using plasmas from high cholesterolemia patients by means of plasma exchange therapy, we selected dextran sulfuric acid as the most appropriate ligand.

In the case of the other constituent element, support, it plays a decisive role in determining the adsorbent's practical performance, for example, its adsorbing capacity and its adsorbing speed. It is duly expected to be provided with the following features: 1) the support itself has little nonspecific adsorption, 2) it can withstand sterilization, and 3) functional groups for embedding ligands can be easily introduced into it. Besides being provided with the aforementioned features, the support is required to satisfy, from the perspective of its practical use, the following two conditions:

(1) It is provided with giant pores that run deep inside its interior so that LDL which is made up of giant particles can penetrate deep into its interior.

(2) It has ample mechanical strength to permit highly fluids such as blood plasma to flow on it at relatively high speeds.

To satisfy the former condition the support is ideally a rough porous material, while to satisfy the latter condition the support is ideally made of a dense material high in solid density. The two conditions are contradictory. Thus, the choice is very limited. We selected a porous cellulose bead as the support that satisfies the above conditions and features. By fixing on the bead dextran sulfuric acid by a powerful covalent bond, we obtained our desired adsorbent (LA-01).

Evaluation of Adsorbent

The results of our evaluation in vitro of the adsorbent's selective adsorbent using human plasma revealed that LA-01 has a high level of affinity for LDL and VLDL, that it scarcely adsorbs HDL, and that it has little effect on the density of major proteins and electrolytes in blood plasma. In animal tests using WHHL rabbits using a small column, LA-01 proved its selectivity and its high LDL adsorbent-removal capability.

Clinical Applications

By filling a polycarbonate vessel (400 ml) with LA-01 and subjecting it to high-pressure steam sterilization treatment, we obtained a column for clinical use. Using the column in combination with a hollow fiber plasma separator made of polysulfone, we treated high cholesterolemia running in

the family. The results of the first treatment revealed that the density of LDL in the blood dropped to less than half the initial value, that little drops in the density of the major blood plasma proteins including HDL were barely observed, and that the system is effective for treatment of hard-to-cure high cholesterolemia.

Article by Noboru Inoue, assistant director, Social Insurance Central Hospital: "Toxic Substances That Increase in Liver Dysfunction and How To Remove Them" [pp 46-47]

1. Foreword

There has been a tendency of an increased number of cases in which liver dysfunction leads to death. Since it is yet unknown how the disease progresses, no treatment methods have yet been established. The problem is becoming an important clinical task. In recent years, a blood cleansing treatment based on circulation of blood outside-the-body has been used to treat liver dysfunction and some successes have been reported.

The objective of the blood cleansing is to remove pathogenic substances that increase in liver dysfunction and correct the metabolic abnormalities. But, identification of the pathogenic substances or toxic substances has yet to be fully established, and consequently the treatment technology has yet to be fully developed. As a result, the treatment records have been far from satisfactory.

The objective of this research is to identify the toxic substances or pathogenic substances that increase in the blood of liver dysfunction patients and develop the corresponding blood cleansing technologies.

2. Research Method

1) Development of the selective removal method

In an effort to develop a technique that enables us to separate substances in the blood according to the size of their molecular weight, we trial manufactured various films with different pore diameters and used some of them in clinical applications. As a representative substance which in case of liver dysfunction increases but which is hard to normalize because of large storage capacity in the body and strong protein bonding, we selected bilirubin in order to conduct a basic study to develop specific adsorbents.

2) Analysis of toxic substances

Using the technology of high-speed liquid chromatography, we fractionated plasma of liver dysfunction patients according to the size of the molecular weight, and then proceeded to determine chemical properties and simultaneously conducted toxicity tests using cultured liver cells.

3) Clinical applications

We conducted the plasma exchange treatment using about 5 l of fresh frozen plasma once every day and tested continuous blood filtration using a newly developed blood filtration film.

Then, we studied the treatment's effects on removing the toxic substances in the blood.

3. Achievements

1) New membrane module

Using a hollow module made of the newly developed polysulfone film, we examined its blood and blood plasma filtering capacity from how well it had managed to prevent passage of various elements. According to our results, our hollow module showed an extremely sharp separation capacity compared with any other hollow module made of film.

2) Analysis of toxic substances

Specific substances of intermediate molecular weight (about 1,000 to 1,500 in molecular weight), were detected in the blood plasma from liver coma patients. They could be removed by either blood filtration or circulation of blood (blood plasma) through activated charcoal.

Peptides were confirmed in the chemical determination experiment but the identification experiment itself was a failure.

3) Clinical applications

We administered continuous blood purification treatment that consists of plasma exchange and blood filtration in five cases of severe hepatitis. The treatment was very effective for hard-to-cure liver coma and improvement in the recovery of consciousness was observed in four out of five cases. However, the life saving rate was low--two of five cases.

4) Considerations and conclusion

Among the basic strategies for treating liver dysfunction are:

- 1) correcting metabolic abnormalities;
 - 2) preventing the progress of necrosis of the liver cells;
 - 3) promoting regeneration of the liver;
 - 4) removal of the causes such as virus; and
 - 5) countermeasures against complications.
- Introduction of blood cleansing technology has enabled us to correct metabolic abnormalities but its effects on liver regeneration and on preventing the progress of cell necrosis are not yet certain.

The plasma exchange therapy used in combination with the continuous blood filtration method was highly effective in correcting the imbalances of amino acids which are stored in the body in large amounts, and the treatment corrected metabolic abnormalities in advanced liver dysfunction.

The newly developed small-size blood filter showed no deterioration in performance and had a good biocompatibility, and it withstood continuous 7-day use.

Article by Koichi Kojima, chief, Biochemistry Research Office, Chemical Research Department, Hatano Research Laboratory, Food and Drug Safety Center: "Basic Research on Application of High-Performance Affinity Chromatography to Medicine" [pp 48-49]

Not a small number of cases have been seen in which substances produced as a result of malfunctions of the organs, tumors, metabolic abnormalities, or immune abnormalities accumulate in the blood, leading the living body to suffer from a serious condition. The substances that cause such symptoms show a variety of molecular morphology. In order to treat diseases believed to be caused by such harmful substances, it is necessary to remove those harmful substances from the blood. Various kinds of dialysis films and adsorbents have been developed and improved for practical use. However, because of imperfectness of films and adsorbents, there have been cases where they failed to display functions expected of them, or where they have led to side effects, and moreover, new harmful substances have been discovered. In order to solve these problems, the demand for development of filters with much higher selectivity or specificity and of adsorbents with a higher specificity has increased, and active efforts have been made toward that goal. In this research, we aim to obtain basic knowledge on how to further increase the sophistication of affinity chromatography expected to have a relatively higher selectivity and specificity and on its applications in medicine.

It is difficult to say what approach we are going to take in our effort, but one way will be to construct a new method of affinity chromatography in which harmful substances are specifically removed but necessary substances are not removed. Conventional affinity chromatography techniques have mainly been developed to efficiently obtain the target substances. Conversely, these techniques can be expected to be put to use for efficient adsorption and removal of harmful substances. Another approach is toward further sophistication of the affinity chromatography technology. Such a sophisticated technology can be used as a new analysis means. A new analysis method with increased performance would enable a high-level separation within a short period of time and could make it possible to detect the target substance specifically and yet with high sensitivity.

With dopamine β -enzyme hydroxide (DBH), β_2 -microglobulin (β_2 -MG), and prealubumin (PA) as our models, at present we are conducting basic research on them.

DBH plays an important role in the metabolism of catechol amine, a neurotransmitter. Innerved by sympathetic cells, DBH, it is believed, is discharged outside of the nerve cells from reserve granules by the mechanism of exocytosis and in the peripheral tissues the greater part of it is transferred into the blood via lymphatic systems. Measurements of the amount of DBH in the blood provide a guideline to the activity of the sympathetic nerves, and the amount of DBH in the blood is also drawing

attention in the field of research on the crisis mechanism of high blood pressure. It is, for example, known that a dose of an anti-DBH drug prescribed has the effect of lowering the patient's blood pressure. If studies of alterations in the DBH activation in various cases lead to its application in medicine, an important means would be to purify DBH and learn its characteristics. DBH proteins are of a membrane-binding type and a free type, and furthermore compared with other tissues purification of DBH in the brain or in the blood entails much difficulty. So, we manufactured an immunoaffinity column and examined the differences in its adsorption and desorption capacity by the differences in the type of antibodies to be combined as ligands. Using this column, we also purified the DBH in the blood serum. Furthermore, using the enzyme immunoassay (EIA) developed by taking advantage of the antibodies, we measured the amount of this enzyme in the cerebrospinal fluid of Parkinson's disease patients.

Among patients who have been receiving dialysis treatment for long periods of time are reported a symptom called amyloid sedimentation. In cases of the symptom, β_2 -MG has been reported as one of the precursors to the substances accumulated. It has also been reported that in some of other amyloidoses, there is observed in their deposits a substance that immunologically reacts specifically to the anti-serum of PA. We are also conducting basic research on how to put the two proteins to good use, such as development of the EIA measuring methods and manufacture of immunoaffinity columns.

Affinity chromatography has until now been scarcely used in medicine. But, once the technology of high-performance affinity chromatography has been established, it would make it possible to easily remove the targeted harmful substance by means of specific affinity and would also make it possible to isolate the targeted substance with ease, thus possibly opening the way for the study of the substance's nature, especially its pathological nature. The use of the high-performance affinity chromatography would also make the isolation and identification of substances in the body possible, a feat that has been difficult with the conventional method, and thus could contribute to accurate diagnosis of disease, to probing the causes for disease, and to determination of the effects of therapy. For these reasons alone, basic studies on high-performance affinity chromatography are needed.

Article by Takeshi Takeda, chief, Pharmaceutical Department, National Institute of Health: "Release-Controlled Drugs and Evaluation of Their Raw Materials" [pp 50-51]

In the development of drugs in recent years, greater interest has been placed on the forms of dispensing drugs that will increase the effectiveness, safety, and usability of existing drugs as well as on development of new drugs. The objective can be found in how to control the release of drugs and how to send drugs to the target, and to that end, studies have been done on new materials including polymers. But for excellent forms of drugs to be developed, it is absolutely necessary to establish rational methods for evaluating the drugs and their materials.

No satisfactory evaluation methods have yet been established, and there still remain many problems to be studied. This research was started aiming at establishment of rational evaluation methods of release-controlled drugs and their materials, and the following describes some of our research results.

In the trial manufacturing stage of release-controlled drugs, experimental animals are often used to evaluate speed characteristics. In this case, full care must be paid to the differences between humans and experimental animals. There have been only a few cases in which studies have been done on factors that have different effects on speed characteristics of drugs between different species. We, therefore, decided to study moving characteristics inside the digestive canals that have a great impact on adsorption of agents of orally administered drugs, and to that end, we compared tablets and granular drugs as to the speed by which they are discharged from the stomach. Dogs had the fastest discharge speed, followed by humans and miniature pigs. Domestic rabbits had an extremely slow discharge speed. From our experiment, it is obvious that in the evaluation of orally administered drugs, the differences in the discharge-from-the-stomach speed that exist between different species must be taken into account. We then examined the effect of food on discharge and adsorption of chemical agents contained in slow-releasing orally administered drugs. In an experiment using dogs, the bioavailability of drugs given after a meal was much lower than that obtained when the drugs were given after the animals fasted. It has become apparent that in evaluating slow-releasing orally administered drugs, the effect of food must be taken into account.

As an analysis method for testing drugs, liquid chromatography is frequently used. So, we examined how the analysis time could be shortened by means of signal processing by Kalman filter. As a signal processing algorithm, Kalman filter has been used in smoothing and peak separation, and differing from the least square method and the Fourier transform method, it can obtain estimated values in real time. When a sample is poured into the liquid chromatograph at short time intervals, a signal with a pile-up of chromatograms is obtained. In order to process the piled-up signal, we established a Kalman filter-based analysis system using a microcomputer.

Using the system, we did an analysis by the convolution process, and the measured values thus obtained were as accurate as values obtained by ordinary measuring, thus showing the system's capacity to shorten analysis time.

Among materials drawing attention as raw materials for release-controlled drugs are biodegradable polymers. Detailed analysis of in vivo degradation behavior of the macromolecules is an important task for evaluating their use as drug materials as well as for evaluating the drugs produced using such materials. So, as a simple measuring method of detailed distribution of molecular weights which is necessary for a detailed analysis of high polymers, we introduced a system combining gel-permeation chromatography with a low-angle laser light scattered light photometer (LALLS) and

examined its adaptability for use in tracing the degradation behavior of high polymers. First, we established a personal computer data analysis system to obtain the molecular weight distribution of high polymers. In this system, temporal deviations of signals from the LALLS and the differential refractometer are compensated for, weight-averaged molecular weights in each of the dissolution times are obtained from the two observed values, and a dissolution time-molecular weight curve is plotted by a higher-order recursion. The molecular weight distribution is determined based on the curve.

Using sodium polyglutamate as a model high polymer, we studied its degradation behavior by enzyme in the established LALLS system. As for changes in the molecular weight distribution accompanying degradation by enzyme, α -chymotrypsin which is endopeptidase and carboxypeptidase B which is exopeptidase showed different patterns, showing the system's effectiveness in the analysis of degradation behavior of biodegradable polymers.

Based on these findings, we are advancing studies on the rational evaluation methods of release-controlled drugs and their materials.

Article by Yoshihito Ikada, professor, Medical High Polymer Research Center, Kyoto University: "Slow-Releasing Protein Drugs" [pp 52-53]

Some protein drugs produced by means of genetic engineering have already been applied clinically. It is anticipated that beginning with enzyme drugs, various proteins will be developed as medical drugs in the future and they will be put to clinical applications. If these protein drugs are based on proteins derived from animals other than man--thus proteins different from human proteins--it will be necessary to deprive those protein drugs of their antigenicity and immunogenicity. But, even in the case of human-derived proteins, when injected directly into the body, they stay in the blood in high concentrations for an extremely short period of time. How to extend the duration time is a great task for now. Well-known methods for the purpose are chemical modification of protein and encapsulation of protein in a high polymer. The former is mainly used to raise the stability in the blood of protein, and the latter is employed for the main objective of slow release of protein drugs.

In the paper the author describes the slow release of drugs. It has been reported that it is possible to slowly release protein even when using a nondegradable type of high polymer matrix like ethylene-vinyl acetate copolymer, but the most common methods are to confine protein inside a liposome or to encapsulate protein in a biodegradable high polymer matrix. Liposome is unstable in the blood, so it is not used for slow release of protein.

We selected urokinase, insulin, interferon, and TNF as protein drugs, and we are promoting studies on how they can be made to release slowly or how they can be made to proceed directly toward their targets. As high polymer support agents, we are using glycolic acid-lactic acid copolymer, gelatin, and polycyanoacrylate. Although we have been using polyethylene glycol as

a synthetic catalyst for binding enzyme, we have not used it in enzyme drugs. Protein is water soluble while glycolic acid-lactic acid copolymer is oil soluble, so some ingenuity is needed in order to mix the protein evenly in the matrix. When protein drugs were encapsulated in degradable fine particles, depending on the manufacturing condition of the fine particles bursts at early stages or denaturing of the proteins were observed. We are also conducting studies on not fine particles of several micron in size but much larger sized pellets, and conversely on extremely fine water-soluble drugs. At this meeting we are going to report on the results we have obtained in our research on slow-releasing protein drugs.

Article by Koji Igarashi, Tokyo Research Center's Bioengineering Laboratory, Toso Co., Ltd.: "Development and Improvement of Signal Sensitive Membrane" [pp 54-57]

Conventional studies on controlled release of drugs have focused on the research on sustained release of drugs, a drug delivery system in which effective amounts of drugs are released slowly over a long period. But, there has been recent research on what the term "controlled release" means. That is, research has been conducted on a system that will ensure that the required amount of drug is released as needed. Figure 1 shows a conceptual diagram of an artificial organ, which we are trying to develop, that combines a pH sensitive membrane with glucose oxidase and that is capable of controlling the amounts of insulin discharge according to changes in the blood sugar level. This time, we have succeeded in developing the system's most important constituent element, signal sensitive membrane. The device is described as follows.

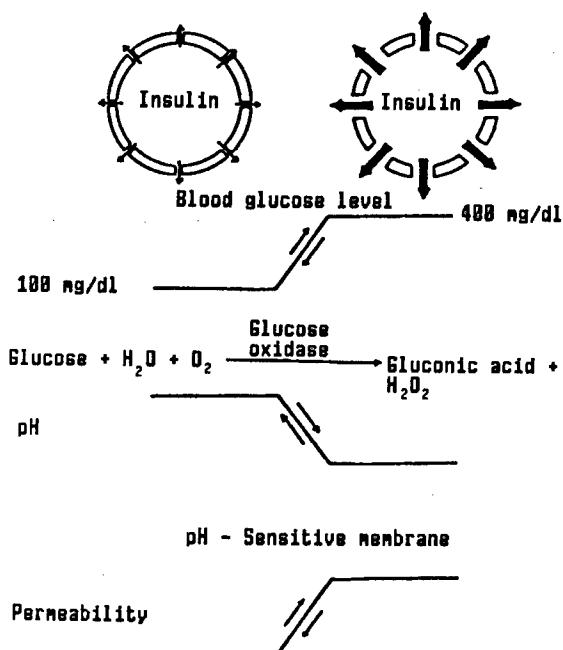


Figure 1. Conceptual Diagram of Insulin Delivery System

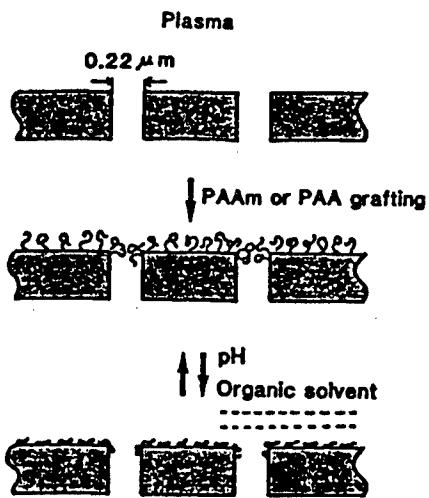


Figure 2. Manufacturing Method and Working Mechanisms of Signal Sensitive Membrane

Figure 2 shows the manufacturing method of this signal sensitive membrane and its working mechanisms. A porous membrane made of polyvinylidene fluoride is grafted with polyacrylamide (PAAm) or polyacrylic acid (PAA) by plasma pretreatment surface graft polymerization. The graft chains on the porous membrane are in a solute state on the porous membrane surface under proper conditions. When they plug the pores of the porous membrane and thus slow its filtration speed, this gives the porous membrane an ultrafiltration capability. When the solution turns into a state of poor solvent for the graft chains, this is reflected as smaller spread dimensions of the graft chains. The shrunken graft chains help unclog the membrane pores that were covered with the solute graft chains, increase the membrane's filtration speed, and rob the membrane of its ultrafiltration capability.

As an example of environmental change, we have plotted data in Figure 3 which shows the effects of pH on the filtration characteristics of PAA graft membrane. In the acid domain where pH is below 3, the filtration speed is fast, and the membrane does not have an ultrafiltration capability, allowing even Blue-dextran with a molecular mass of 2 million to flow through it. When the pH level is gradually raised, in the area of pH 3 to 4 the filtration speed slows down sharply and the membrane gains its ultrafiltration capability. When the pH was above 5, the removal rate of Blue-dextran reached 100 percent.

The PAA graft membrane functions as a pH-sensitive membrane. A problem with it is that since the pH of living bodies is in the neighborhood of 7.4, if the membrane is to be used as an artificial organ, its pH level, in which its filtration characteristics change, is too much in the strong acidity range.

Therefore, in order to adjust the pH of pH sensitive membranes, we prepared a high polymer amphotolyte graft membrane. By letting a PAAm graft copolymer

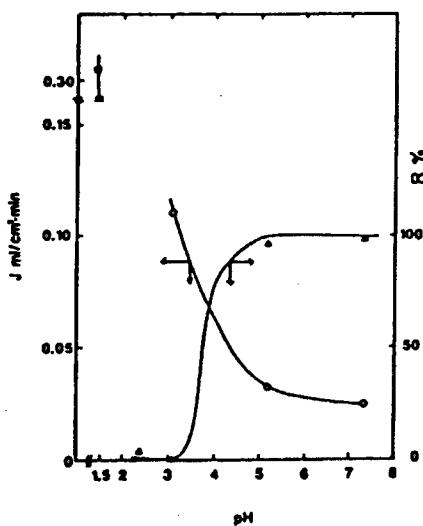


Figure 3. Effects of pH on Filtration Characteristics
of PAA Graft Copolymer Membrane
Pressure applied: 2 kgf/cm²

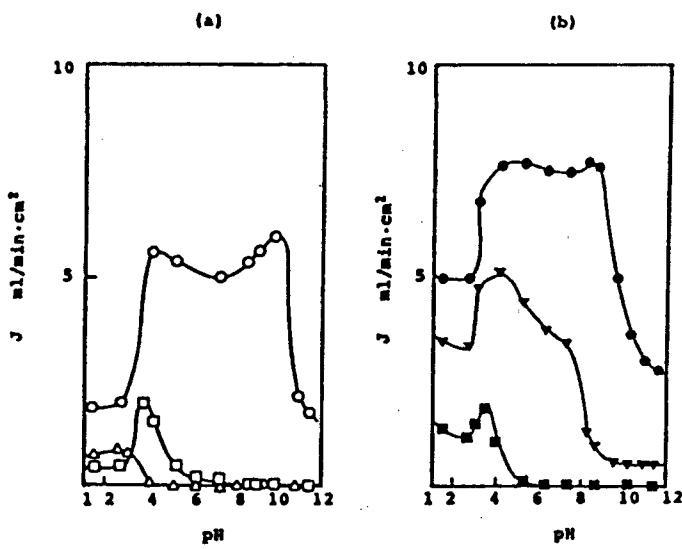


Figure 4. Effects of pH on Filtration Speed of Copoly-AA-VAm Graft Copolymer Membrane

Pressure applied: 0.6 kgf/cm²

(a) Graft copolymerizing time 30 min.

Hoffmann reaction time Δ : 15 min; \square : 30 min; \circ : 60 min

(b) Graft copolymerizing time 60 min

Hoffmann reaction time \blacktriangle : 30 min; \blacksquare : 45 min; \bullet : 60 min

membrane go through a Hoffmann reaction, acrylamide is converted into vinyl amine (VAm). The remaining amide residues are subjected to hydrolysis to obtain acrylic acid, and we obtained an acrylic acid-vinyl amine copolymer (copoly-AA-VAm) graft copolymer membrane.

Figure 4 shows the pH response characteristic of the obtained copoly-AA-VAm graft membrane. It has regions of lower filtration speeds on both sides of the higher temperature region and the lower temperature region. Between the two extremes, in the regions with higher temperature levels the carboxyl groups in the graft chains have negative charge while in the regions with lower pH levels the amino groups are positive. So, in the pH regions on both sides the graft chains spread wider, causing them to close the membrane holes. Comparison of various copoly-AA-VAm revealed that as the Hoffmann reaction time becomes longer, because the amounts of VAm in graft chains increase, the pH domain that triggers a change in the membrane's filtration characteristics shifts to the alkaline side. By adjusting the duration of the Hoffmann reaction time, it is possible to prepare pH-sensitive membranes that greatly change their filtration characteristics in the neighborhood of pH 7.4, the value equivalent to pH of living organisms.

We have yet to develop a glucose oxidase embedded membrane, but we feel that we have gained a foothold in the development of a system that can slowly release the required amount of insulin as needed in response to a change in the blood sugar level.

Article by Ken Murayama, leader, General Research Laboratory, Toyobo Co., Ltd.: "Development of High Polymer Membranes With Antithrombus and Gas Permeability Properties Over a Long Period of Time, Their Improvement Technology, and Their Evaluation" [pp 58-59]

1. **Foreword:** Membrane-type artificial lungs currently used for circulation outside the body and ECMO (Extracorporeal Membrane Oxygenation) have poor blood compatibility and they need to be accompanied by anticoagulation treatment such as heparin, and as a result, patients tend to suffer from excess bleeding. Their gas exchange capabilities also suffer damage over a certain period of time and there is also the problem of plasma leakage (wet lung). For these reasons, they can be used for 3 days at the longest, the poor blood compatibility of these artificial lungs is caused by the blood compatibility of the materials being used in the artificial lung sections and the plasma leakage is caused by the gas exchange mechanism. Even with the membrane type artificial lungs rated to have the highest levels of blood compatibility, there still remain problems in formation of thrombi or complement activation. Consequently, to develop an artificial lung that can take on the function of the lung over a long period of time, we believe the blood compatibility of the membrane material definitely must be improved.

The objective of our research is to develop high polymer membranes that can be used without heparin and that, besides being capable of inhibiting complement activation, can retain antithrombus and gas-permeability properties over a long period of time, and to develop an artificial lung system that can truly be used as an artificial organ using those membranes.

2. Synthesis of membrane material for artificial lung that has blood compatibility

The membrane material for artificial lungs that satisfies our research objective must be provided with the following three conditions:

(1) Has excellent blood compatibility (antithrombus and complement activation inhibition).

(2) Has high gas permeability (of oxygen and carbon dioxide).

(3) Has excellent morphology-retaining capability (can be easily fabricated into thin films or can be formed into a hollow shape, and moreover has high strength).

Furthermore, if wet lung is to be prevented and if the gas exchange capability is to be maintained over a long time, the membrane will have to be of a nonporous type membrane (uniform membrane) structure. Polydimethylsiloxane (PDMS) can be fabricated into a uniform quality membrane yet has sufficient O₂/CO₂ permeability. The material's shortcomings are that it is low in strength and can hardly be fabricated into thin films.

From the foregoing perspective, in order to obtain a PDMS-based material which is strong enough to be fabricated into thin films and which has blood compatibility while keeping intact the PDMS' excellent gas permeability, we synthesized on a trial basis a block copolymer (PDMS-PU) consisting of amorphous and hydrophobic PDMS segment and crystalline and hydrophilic polyurethane (PU) segment.

It was found that the polymer's oxygen permeability was dependent on the content of PDMS in the PDMS-PU and that the PU segment had nothing to do with the oxygen permeability.

That is, since oxygen selectively passes through the PDMS segment, it has been suggested that the purity of the microphase separation structure between PDMS and PU segments has an effect on the oxygen permeability. Consequently, one of the future tasks is to study the effects of the domain size and purity in a microphase separation structure on the gas permeability and blood compatibility.

The PU segment content, on the other hand, has a great influence on the material properties. With increases in the amount of PU, the strength of the block copolymer and its Young's modulus increase exponentially. Consequently, its morphology-retaining and workability properties increase, and we believe that the loss in gas permeability in the PU segment can be compensated for by fabricating the hollow fibers and membrane more thinly and that as a result, PDMS-PU can attain a de facto higher gas permeability than PDMS alone that can hardly be fabricated into thin films.

3. Conclusion

(1) We searched for a block copolymer having both gas permeability and blood compatibility and tested the PDMS-PU block copolymer system. We succeeded in the synthesis of a membrane material provided with full material properties.

(2) In the PDMS-PU system, the PU segment has hardly any effect on the oxygen permeability and its $P(O_2)$ drops than that of PDMS alone. But, thanks to its increased strength and moldability, PDMS-PU can be fabricated into thin films. We believe the PDMS-PU system has a greater transmission in reality than the membrane of PDMS alone which is hard to fabricate into thin film.

(3) After thoroughly studying what effects changing the amount of PDMS or introducing different segments will have on blood compatibility or gas permeability, we plan to conduct studies on the hollow fiber-making technology, thin film-making technology, and coating technology and to develop the material we have been aiming for.

Then we plan to manufacture an artificial lung system using the material and compare it with other similar devices.

Article by Takehisa Matsuda, chief, Bioengineering Department, Research Laboratory of the National Cardiovascular Disease Center: "Development of a Silicon Elastomer for Medical Use and Evaluation of Its Antithrombus Properties" [pp 60-61]

1. Objective

Required of a material for artificial organs to be used in the blood circulation system are 1) its dynamic properties as an elastomer; and 2) its antithrombus properties. As a material that satisfies the two performance requirements to the greatest common measure, segmentized polyurethane has been used as the basic material for artificial organs of the circulation system represented by blood pumps. Theoretically, by choosing different types of starting materials, various types of segmentized polyurethane with different surface features and different dynamic properties can be synthesized. Kanegafuchi Chemical Industry Co., Ltd., to which the research project has been commissioned, has been engaged in the development of a type of segmentized polyurethane that uses silicon-polyethylene glycol block copolymer as an element of its soft segment at its central research laboratory for years and has succeeded in developing an excellent antithrombus elastomer for medical use (KP-13).

In this research, we first analyzed in detail by X-ray photoelectron analysis (ESCA) the composition and distribution of KP-13's surface layer, several tens of angstroms thick, which govern the material's blood compatibility, then proceeded to evaluate its in vitro antithrombus properties from major living organism systems regulating whole blood coagulation properties (Lee-White whole blood coagulation time) and blood coagulation as coagulation, complement system activation, and cell-binding

properties, and finally researched the factors governing responses of the human body in relation to the surface composition.

2. Research Results

Analysis of the surface's microstructure (KP-13)

ESCA can give data on the chemical construction and bonding state of the surface layer, about several tens of angstroms deep. An analysis of KP-13 using the technique revealed that no N atom deriving from the hardware element was observed in the surface layer of up to about 10 Å that governs the material's blood compatibility and that the hardware element was exposed to the far outer surface. On the other hand, polyethylene glycol was observed partially exposed to the surface, but the majority of the surface was covered with silicon.

Evaluating biocompatibility

As part of the in vitro antithrombus evaluation, we compared our KP-13 with six types of medical polyurethane that have already been commercialized or are under development at home or abroad in such fields as whole blood anticoagulation (Lee-White whole blood coagulation time), activation and cell-binding properties of the coagulation and complement systems. In measuring Lee-White whole blood coagulation time, KP-13 had an edge over others in terms of the relative whole blood coagulation (average value of n=6). KP-13 did not give rise to adsorption and activation on the part of the complement system. In the coagulation system, it also invited an extremely small level of activation. When these results are taken into account, KP-13 has excellent anticoagulation properties to whole blood coagulation and protein biosystems. Adsorption and activation of platelets on the surface of material is one of the major pathways to formation of blood clots. We have discovered that ligand-receptors at adhesion sites of fibronectine and fibrinogen play an important role in the adhesion and activation of platelets. On the other hand, we have also discovered that the similar ligand-receptor interaction plays a controlling role in the case of adhesion of endotheria. As a result, observing the adhesion and multiplication behavior of endotheriums would enable us to quantitatively estimate the strength of their interactions with platelets at the levels from hours to days. Compared to other types of polyurethane, KP-13 has proved to be able to completely prevent adhesion and multiplication of endotheria, and thus it is considered to have excellent plugging effects of platelets.

This nonadhesiveness is considered to have been caused by the fact that as a result of the condensation of the hydrophilic polyethylene glycol chains on the boundary with water, adhesive proteins like fibronectine were subjected to adsorption inhibition. Consequently, the surface composition of the membrane formed in the air is hydrophobic, but the surface tissue in the actual use environment of a polar field is highly hydrophilic, and this strongly suggests that there has occurred an inversion of the surface tissue in the water environment.

Article by Katsuyuki Miyasaka, chief, Pathologic Physiology Research Office, Pathology Department, National Children's Hospital Pediatrics Medical Research Center: "Examination and Evaluation of Antithrombus Treatment by Cardiac Output Continuous Catheter" [pp 62-63]

To measure the cardiac output of advanced circulatory insufficiency patients--an essential measurement for such patients--a thermal dilution catheter usually placed in the pulmonary trunk is used. However, with existing methods measurements cannot be taken continuously and the procedures require injection of a large amount of cold, raw salt water and thus the load on circulation cannot be ignored. Furthermore, because the possibilities of low body temperature and infection by virus exist, the number of measurements that can be taken is limited. Aiming at continuous monitoring of the cardiac output without imposing any added burden on the patients, we have developed a method to continuously measure cardiac output, in which the thermal losses are continuously measured by additional improvements on the ordinary thermal dilution method and changes in the flow speed of blood in the pulmonary trunk. There are many methods to continuously measure cardiac output, but there has yet to be established any method that can be used in clinical applications on a stable basis.

Attempts to improve the thermal dilution catheter are acceptable from the clinical viewpoint, and there have been many trials to continuously measure cardiac output. The majority of these trials involve a process in which blood is heated using a heater and changes at the downstream are measured. At the downstream, however, the necessary heating energies sufficient to sense changes are reported to be as large as about 4 W. Based on the principle of thermal loss measurement, and using a method of continuously detecting changes in the quantity of heat lost to blood flow using two thermistors, our method consumes only 100 MW. Furthermore, for easy calibration we have retained the conventional thermal dilution catheter function, thus making it possible to minimize the heat burden, and in principle, the method enables measuring over a long period of time.

Although our method has made it possible to continuously measure cardiac output, differing from conventional methods it is accompanied by heating of the thermistors. In this respect, studies will have to be done on problems which will arise when keeping a catheter in the pulmonary trunk for a long time, especially how to impart antithrombus properties.

This time, we used our cardiac output continuous measuring catheter (KATS catheter) in animal tests to evaluate its performance and at the same time conducted basic studies on antithrombus capabilities of HS-60PVC, a device manufactured for imparting antithrombus capabilities that has PVC as the basic material and polyhydroxyethyl-methacrylate-polystyrene block copolymer (HS) coatings.

The HS-60 coating was confirmed to impart antithrombus capabilities in both *in vitro* and *in vivo* experiments, giving hope for its application to the cardiac output continuous measuring catheter. The HS-60 being used at present, however, has rather rigid material properties, so we believe that it can be fully applicable for coating the catheter but that it has

problems when applied to the balloon portion made of soft latex. We feel further studies must be done on methods of imparting antithrombus capabilities to soft and flexible materials, as well as on the effect a material has on the action of measurement itself and on a way to make the HS-60 softer and more flexible. We have described the basic performance of the continuous cardiac output measuring catheter and on findings we have so far obtained on antithrombus capabilities.

Article by Hiroyuki Nakazawa, chief, Analytical Chemistry, Hygienic Pharmacology Department, Institute of Public Health: "Basic Studies on Blood Compatibility by Faint Luminescence Phenomenon" [pp 64-65]

1. Foreword

We can observe around us various light emission phenomena represented by such things as bioluminescence by luciferin-luciferase and chemiluminescence by luminol. By measuring the light produced by chemical or biochemical reactions, not only have specific analysis technologies of the substances involved in the reaction been developed but also the mechanisms of the reaction have been shown. These technologies are finding wide application in various fields from food to medicine and chemistry, from environment to clinical chemistry.

On the other hand, contributing to the high-level medical technologies that have seen rapid advances with an aging population has been development of various polymer materials for medical use. These medical materials are required to have not only mechanical strength and chemical stability but also excellent compatibility with the body. To evaluate blood compatibility, one of the most important of all, various *in vivo* or *in vitro* antihemolysis or antithrombogenic materials tests have been developed. These test methods involve observation of the reaction between blood and material. In this research we plan to use the faint light measuring technology that has advanced greatly as a result of advances in electronics technology in the measurement of the reaction and examine the technology's adaptability to evaluation of blood compatibility in the surveys and experiments listed below.

2. Research Plans and Achievements

2.1 Measuring cell-derived light emission

By trial manufacturing a measuring system of extremely faint light emission by a photon-counting method, we measured extremely faint light that is generated at the contacts between neutrocytes, a representative active oxygen formation line, and solid surfaces of such innervational materials as PMA and of medical materials.

On the other hand, one of the tasks in evaluating blood compatibility is the problem pertaining to blood clotting. Therefore, we studied by means of luminescence measurement generation of active oxygen or other radicals when platelets become coagulated or become activated. To clarify the

identities of these light emissions we will simultaneously conduct studies by ESR (spin trapping process).

Furthermore, by taking note of the calcium ion considered to be an essential element in the function of many of the cells, we have analyzed changes of intracellular calcium ion using the luminescent protein, "equorin."

2.2 Effects of active oxygen on blood components

Contacts between blood and medical materials are expected to cause neutrophiles and monocytes to produce active oxygen, and in connection with the formation of blood clotting, the active oxygen is feared to have an adverse effect on the coagulation of platelets. Therefore, we studied if active oxygen produced by a simpler xanthine-xanthine oxidase line has any effect on the coagulation or noncoagulation of platelets and further how the effect of the active oxygen on the coagulation reaction is affected by ADP or collagen.

Blood vessel endotheliocytes, on the other hand, play an important role in the embedding and growth of artificial blood vessels. From research on hardening of the arteries, it has been suggested that blood vessel endotheliocytes may be damaged by white blood cells including macrophages. So, in this research we are going to study the effects of active oxygen and white blood cells on endotheliocytes.

Mast cells react to various kinds of stimuli in the form of degranulation, and they are deeply involved with inflammation. The effect of active oxygen on the reaction is also a subject of interest.

2.3 Basic studies on related cells

To understand reaction mechanisms between various kinds of cells and medical materials, it is necessary to understand the reactions of individual cells. So, we intend to show at a molecular level the mechanisms of such reactions as blood coagulation and production of active oxygen by such means as studies on the behavior of calcium ion using various chemical agents, measurements of thrombomodulin and related factors, and analyses of protein phosphorylation reactions.

This research has been undertaken as a joint research with the Institute of Public Health (Nakazawa, Sumiko Suzuki, Masahiko Fujita), the Food and Drug Safety Center (Hiroshi Ono, Nobuyuki Ogawa), Irika Kogyo Co. (Hisao Tsuruta, Ichiro Matsuda), the Dental Faculty of Tokyo Medical and Dental University (Ikuo Morita), the Faculty of Pharmaceutical Science of Teikyo University (Hidemi Ishi), and Saitama Medical College (Masaru Sonoda).

Article by Kazutoshi Iida, member, Chiba Research Institute, Ube Industries, Ltd.: "Development of Technology for Reforming Polymer Surface and Its Application to Medical Instruments" [pp 66-67]

Among capabilities demanded of medical equipment, especially materials for artificial organs, is blood and tissue compatibility. The compatibility is dependent on the nature of the top surface layer of material, and it is the responsiveness of the body to recognize an alien substance on the interface between body and material.

Consequently, the essential conditions for implant-type artificial organs are to avoid as much as possible activation of the body's defense mechanism at the body/material interface by reducing as much as possible interaction between the material and body fluids and blood components. In this project, using cellulose and segment polyurethane, a polymer, that is used widely as the basic material for embedded type artificial organs of the blood circulation system, we are going to conduct research on the reforming technology of the body fluid/blood component contact surface by chemical modification. Next to be observed are the chemical structure and material properties of the material's polar surface. The next items for examination are biological responses: That is, the adsorption and activation of the coagulation and complement systems that are the body fluid-based defense mechanisms triggered by contact between these reformed materials and body fluid/blood components, and the attachment and activation of blood platelets, another defense mechanism, are to be examined. Our goal is to consider the relationships between the reformed material surfaces and biological responses, to clarify the reforming methods and conditions that will restrain as much as possible activation of the body's defense mechanisms, and thus to probe the possibilities of applying such reformed materials to medical instruments and equipment.

In this report, we shall describe our studies of how to introduce long-chain alkyl groups and polyethylene glycol groups into the base materials of cellulose and polyurethane, as well as results of X-ray photoelectron spectroscopy (ESCA) and contact angle measurements of surface material properties.

Furthermore, we shall describe our findings on biological responses triggered by the contact between the newly reformed materials and the body fluids/blood components. Here, the introduction of long-chain alkyl groups into the materials is aimed at realizing biocompatibility by selective adsorption of albumin, by Eberhart, et al., and the introduction of polyethylene glycol is aimed at realizing on polymer surface a nonadsorption/nonadhesion surface similar to the polyethylene glycol graft chain surface developed by Mori and Nagaoka.

Reforming cellulose was conducted using cellulose film and by means of heterogeneous reaction. TDI was first introduced, and then the film was reacted with alkyl alcohol, alkyl amine, perfluoroalkyl alcohol, and methoxyethylene glycol. In following up the reaction process, analyses by ESCA was an important means. Data on the ratios of elements and the state and distribution of their bonds up to about several tens of angstroms were

obtained, and combined use of the technology with the sample gradient method enabled us to obtain data on anisotropies in the depth direction.

In the reaction between cellulose and TDI, the reactivity differed greatly depending on the solvent used, and when using DMSO solvent, the reaction progressed, it became apparent, quantitatively from the surface layer to the inner layers gradually. In the alkylation that followed, an increase in the nitrogen composition in the vicinity of the surface that indicates concentration of alkylated elements, a lowering in the oxygen composition, and the existence of anisotropies in the depth direction were confirmed. This suggests the polar surface has been reconstituted by the alkylation. When PEG was introduced, no reconstitution as observed in the alkylation was observed. The contact angle was controlled by a bubble process by pumping air in the water. In alkylated cellulose, it was observed the contact angle changed depending on the alkyl chain length. It was judged that the increased hydrophobic properties were not entirely relying on the alkyl chain length but were based on the kinetics of the newly formed chemical structure and that a reorientation shifting in the direction where the interfacial free energy is minimum was taking place.

In reforming segment polyurethane, sodium hydride was used as the base and was made to react to alkyl halide and methoxypolyethylene glycol halide by means of uniform reaction. In alkylation, ESCA showed concentrations on the surface of alkyl chains in proportion to the amounts of chemical modifications. When introducing PEG, on the other hand, the PEG was observed to exist in large quantities in layers deeper than the surface. It is believed this is because in the process of fabricating the membrane by cast process from a solution, the PEG penetrated the interior in order to lower the interfacial free energy. The sample was dipped into water and pulled up. Its contact angle was measured by a drip-drop method, which revealed data showing the formation of a high hydration surface. Reorientation of molecules, which is dependent on the external environment, was also observed.

The foregoing suggest that when reforming the surface of a polymer by chemical modification, appropriate molecular design and synthesis conditions need to be selected after fully taking into account the reconstitution of molecules as a result of the chemical modification and the reorientation phenomenon according to the external environment. In the survey of biological responses at the interface between body and material, which we conducted using the materials mentioned above, results that were considered to be the effects of the chemical modification groups were obtained as expected.

Article by Hideo Yano, chief, Department for Motor Function Disturbance Research, National Rehabilitation Center for the Disabled: "Biocompatibility of Hydroxyapatite and Its Endurance" [pp 68-69]

As a result of worldwide research in the past 10 years, hydroxyapatite has come to be recognized as a promising material for use in the body. In the early stages of its applications to practical use, the material was used for the first time by M. Jarcho¹ of the United States and others for the

formation of the lower jaw and in the dental field. A hydrated calcium phosphate with a composition of $\text{Ca}_{10}(\text{PO})_6(\text{OH})_2$, this material is the same as the main mineral forming bones. Depending on its morphology or surface area or pH, hydroxyapatite is said to show various kinds of behavior in the body but much about its activity is unknown. Empirically, different versions of the same material are said to show greatly different reactions to the body or to have different bone-forming capabilities in particular, depending on their formation purities or the character of the abovementioned material. What made us focus our attention on hydroxyapatite is that by adjusting its character, it can be artificially fabricated into materials that have extremely low levels of toxicity yet have excellent bone-forming capabilities, and that bones in the body and artificially manufactured materials show so-called biological bonding. Basic experiments on these features have already been verified,²⁻⁵ and this year we have obtained approval from the Ministry of Health and Welfare for the material's use in the body.

A series of research results have led us to believe that hydroxyapatite can contribute greatly to the prevention and treatment of aging of bones, and we plan to conduct a series of in-depth studies on the material to show its roles in forming bones.

In line with the above objective, in order to manufacture bone-apatite systems that can be used as bone substitutes and that can dynamically withstand the absorption process of bone seen in the initial stage when apatite has been implanted into a bone, we conducted animal experiments, in which the bone and apatite were fixed in place by Ti screws and a dynamic load was applied after surgery.

Results and Consideration

As for the macroscopic findings and CMR findings of the bone-hydroxyapatite-Ti metal in the tissue, the initial bonding was very good, and there was clearly observed the process of bone-formation in the apatite progressing from the bone side. There was also observed no lag that is usually seen when removing the Ti screw, and this suggested a relatively firm bonding in terms of dynamics. As for macroscopic findings, no local reaction to foreign matter was observed, infiltration of cells such as lymphocytes was within the range usually observed after surgery and there was not observed any rejection reaction by the tissue.

We plan to promote analyses of the microscopic findings and qualitative analyses of the dynamic strengths.

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Article by Mitsutada Nakamura, chief, Medical Products, National Institute of Hygienic Sciences: "Research on Safety Evaluation Methods of Polymer Materials for Embedding in the Body; Especially Ethylene Oxide That Remains in the Lens of the Eyes" [p 70]

We are advancing research on safety evaluation of polymers for use as medical materials from two approaches. The first approach is methods to test if materials for implanting in the body will trigger development of cancer. Many kinds of materials have been confirmed to cause cancer in animal experiments. When implanted in the shape of a film in an animal, this is followed by the onset of cancer in that position. In the case of human beings, however, there has been reported only a single case of cancer, the cause for which has definitely been traced to an implant. This occurred in a person who had a Teflon dacron graft embedded for 10 years. The mechanisms of the onset of cancer in rats have yet to be fully clarified. There are two theories about carcinogenesis--alien matter carcinogenesis caused by physical factors and chemical carcinogenesis brought about by chemical substances. The difficulty in distinguishing one from the other further contributes to the difficulty in interpreting the experimental results. By using materials whose chemical properties are fully known and by confirming the properties of the eluted products, we decided to further study the materials' changes (such as disintegration) even after the experiment was over. Furthermore, we prepared experiment systems to observe the effects of differences in the molecular mass, in the surface roughness, and in the ratio of functional groups existing on the surface, and experiments are now underway. Anxious to advance the existing knowledge and methods a step further, we intend to simultaneously conduct *in vitro* tests of the eluted products, or *in vitro* tests of model compounds.

The second approach is to provide methods for searching for the causes of side effects of medical devices. The intra-ocular-lens (IOL) is the smallest medical implant (about 20 mg). Sterilization is provided using ethylene oxide (EO). PMMA, the material for IOL, is one of the materials which EO has difficulty attaching to or detaching from. Occasionally observed in patients implanted with an IOL are such side effects as hypopyon iritis or attachment of fibrin on the lens, and one theory has it that those side effects are caused by the residual EO. This has not gone any further from a theory because there did not exist a method for measuring trace amounts of EO residing in a single IOL. We have established a method for determining EO if it is contained in a single IOL at rates of 2 ppm or above. We shall introduce details of the method at this symposium. The relationships between residual EO remaining in medical devices and its side effects have been discussed in various fields, and we believe our method of determining trace amounts will find applications in areas other than IOL.

Article by Hideo Isaka, chief, Pathological Research Department, Hatano Laboratory, Food and Drug Safety Center: "Tissue-Damaging and Cancer-Causing Potential of Polymer Materials and Development of Short-Term Assaying Method of Such Potential" [pp 71-73]

1. Objective of Research

Polymerized compounds like plastics, polyethylene, and polyurethane have widely been used as medical materials, but their safety, especially their carcinogenicity when kept in the body for a long time, has yet to be fully analyzed. One of the major causes for the lack of knowledge is that to analyze carcinogenicity of these materials, animal experiments using large numbers of animals need to be conducted over long periods of time. In light of such a situation, the objectives of this research are to study the potential of high polymerized compounds centered on medical polymer compounds to damage tissue cells or to cause cancer and to provide useful data for establishing short-term assaying methods of these damage-causing factors.

2. Research Method and Results

2.1 Harmful effects of high polymerized compounds on tissue cells

In our research we used the NIH3T3 cell that is indispensable to modern cancer research, such as transformation experiments of carcinogenic chemical substances, isolation of cancer genes, studies of cancer viruses.¹ The 3T3 cell is a very useful cell but it is by no means characterized by ease of handling. The first task was to screen serums to pick one to be used in the cell's culture. Using five kinds of lots, we examined the serum's effect on the multiplication of the NIH3T3 cell.

After NIH3T3 cell's semiconfluent multiplication in two T25 flasks which were given by Professor Kuroki at the Cell Bank of the Ministry of Health and Welfare, the cells were dispersed by 0.05 percent trypsin, diluted to one-fourth, and cultured in CORNING T25 flasks for generations. Each flask had D.MEM added with 10 percent of the serum to be examined as the medium, and cultivation was conducted under conditions of 37°C, and 5 percent CO₂-95 percent air. The results revealed that PNB (Mitsubishi) facilitated attachment of the cells to the wall surface and active cell multiplication. In the latest generation, 3×10^4 cells/ml increased to 13.3 times the volume in 5 days. In other CS lots, the majority of the cells were seen floating. Even in the case of cells which were attached to the wall surface of the flask, their attaching powers were weak, and no evidence of their multiplication was observed. On the other hand, we fabricated high polymerized compounds into sheets, placed those circular products, 6 cm in diameter, on Petri dishes and sprayed NIH3T3 cells on them. The end products were cultured on MEM mediums added with 10 percent PNB to examine the cells' morphological features.

2.2 Mutation potential of polymer medical materials

We used the following nine [sic] kinds of samples to test the potential of polymers to trigger mutations. They are polyethylene terephthalate, polyvinyl chloride, polysulfone, cellulose, silicon rubber, polyurethane, alumina, biological activated glass.

As the test reagents for causing mutations we used the following: NADH, NADPH, and G-6-P manufactured by Oriental Yeast Co., Ltd.; the Japanese pharmacopoeial physiological saline solution manufactured by Otsuka Pharmaceutical Co., Ltd.; special-grade reagents manufactured by Wako Pure Chemical Industries, Ltd.

Samples were prepared by the following method. Five grams of sample were added with physiological saline solution prepared under the Japanese pharmacopoeia or 10 ml of DMSO, and the mixture was subjected to vibrations for extraction for 24 hours at a rate of 60 vibrations/minute. The extracted solution was made to pass through a 0.45- μm mesh membrane filter and sterilized. The end product was used as the sample for mutation-causing effect.

One mutagenesis test was conducted as follows: Two strains of virus, *salmonella typhimurium* TA100 and TA98, were used. S-9 Mix was prepared using male rats of the Sparague-Dawley family and golden hamsters of both sexes in accordance with the Ames' method.³ PCB (KC400) was given once into the abdominal cavity at the rate of 550 mg/kg of body weight. Five days later the liver was extracted and homogenized by adding 0.15 M of potassium chloride solution; it was centrifuged for sedimentation at 9,000 Xg and the supernatant fraction (S-9) was obtained. S-9 Mix was obtained by mixing per 50 ml of S-9 Mix the following ingredients: 5 ml of S-9 fraction; 1.0 ml solution (water) of magnesium chloride (0.4 M) and potassium chloride (1.65 M); 0.25 ml of G-6-P water solution (1 M); 2.0 ml of NADPH water solution (0.1 M), 2.0 ml of NADH water solution (0.1 M); 25.0 ml of phosphoric acid-Na buffer solution (pH 7.4) and 14.75 ml of distilled water.

The other mutagenesis test was conducted as follows: In accordance with the plate method by Ames, et al.,^{3,4,5} we poured into sterilized test tubes containing 500, 200, or 100 μl of various test solutions 0.5 ml of S-9 mix or 0.5 ml of Na-phosphoric acid buffer solution (0.066 M), 0.1 ml of cultured solution of *Salmonella* (number of bacteria: about 1×10^8) and 2 ml of soft agar (0.5 percent NaCl, 0.045 mM histidine and 0.045 mM biotin included) kept at 45°C and mixed them. The mixture was poured onto an agar plate, spread evenly, and incubated at 37°C for 2 days and nights. We then measured the number of colonies of back mutant strains that appeared on the plate and examined their mutagenicity tests, two plates were used for a given concentration of each of the samples. The yardstick for judgment was the average value of the number of colonies/plate, and when the number of mutant colonies was larger than the control (the number of natural back mutant colonies), the sample was judged to be mutagenically positive. The control is a value of negative control. Studies will have to be made on

different S-9 Mix according to different animal species and on their extraction methods.

2.3 Carcinogenicity test

We conducted transformation tests developed by Kuroki² on substances found to be positive by the Ames test, using BALB-C3T3 along with S-9 Mix. When mutant colonies were found, they were transplanted into nude mice to see if they developed a tumor. We also conducted tissue tests, and if a mutant colony was found to trigger a malignant tumor, we diagnosed the substance to be positive in terms of carcinogenicity.⁶ We shall describe our findings. It was found in the tests that substances eluted from high polymerized compounds had cancer-causing effects, but as to whether the high polymerized compounds themselves were carcinogenic or the compound ingredients such as curing agents were responsible, we are going to conduct the study next year.

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Article by Konoshin Kawasaki, chief, Pharmacological Microorganisms Department, Osaka Branch of the National Institute of Hygienic Sciences: "Research on Standardization of Limulus Tests and on Safety Evaluation of Polymer Membranes and Materials [pp 74-75]

Current medicine demands large amounts of transfusion solutions, injection solutions, and dialysis solutions, and for the production of water use in these solutions various polymer films such as the reverse osmosis membranes (RO membranes) and ultrafiltration membranes (UF membranes) have been widely used. Needless to say, these membranes and membrane modules and water for injection solutions, as well as the drugs thus produced must be free of endotoxin. Recently, water filtered by these membrane modules is about to be approved as the feedstock water for use in injection solutions under the Japanese Pharmacopoeia, and consequently establishment of testing methods for endotoxin (ET) is becoming an urgent task.

Conventionally, the test method for thermogenic substances using rabbits has been used as the test method for ET, but recently a high-sensitivity

detection method using elements extracted from blood corpuscles of horseshoe crabs has come to be widely employed as the limulus test (LAL test).

From the viewpoints of medical practice, solutions of such problems as elution of LAL test positive substances from cellulose-based dialysis membranes, anaphylaxis-like shock caused by "caprofan" hollow dialysis machines, removal of ET from dialysis solutions, and how to ensure strict ET checks of a large number of medical devices used directly in the body and their materials are becoming an important task.

In light of such a situation, we believe establishing the standards for LAL tests to detect trace amounts of ET and then establishing the methods for evaluating ET-removal capacities of polymer membrane modules are important and urgent tasks. To that end, we conducted the following research.

1. To standardize LAL tests, studies need to be made on the following: endotoxin-free conditions for LAL tests, stability of LAL reagents when kept in storage, problems associated with testing methods such as availability of appraising activity of ET, establishment of standard ET products which are necessary for tests, and their comparison with USP standard products, and interrelation among the three methods of the gel method, nephelometric time analysis method, and synthesis matrix method.

1) The results of our research on how an ET-free state can be obtained under what condition revealed that LAL tests become negative after heating at 25°C for 90 minutes and that various types of LAL reagents can be kept in storage at temperatures of -20° to -30°C within 7 days after dissolution, but for preservation for longer periods "toxicolor" and (endospecies) need to be preserved at temperatures below -40°C.

2) At present, *E. coli* UKT-BFT is scheduled to be used as the standard Working Reference ET (WRE). The results of our evaluation of this ET's heat generation and limulus activity and of our comparisons of this ET's activity with that of other ETs, especially the USP standard ET (EC-5), revealed that there was little change in the heat generation and limulus activity of this WRE from their measured values in 1983 and 1986. But there existed differences among various LAL reagents in their sensitivity to WRE. WRE: EC-5 were compared for their potency, and the results were 1 ng:10 µ/kg in terms of the PD₅₀ value and 1 ng:6-10 EM/ml in terms of the limulus activity.

3) There were observed relatively good correlation between the gel, nephelometric time analysis and synthesis matrix methods. However, there were observed big differences between various ETs in their activity.

From the results of these experiments, we believe that to standardize LAL tests, it is important to compare in advance the activity of the ET to be used with WRE and have a clear definition of its activity.

2. In order to evaluate the capacities of polymer membrane modules to remove ET and others, we subjected them to LAL tests and "the number of live virus" tests using the leak of ET and virus as the guide. The results

were that there were observed leaks of live virus in some membranes. Therefore, to further examine their ET and live virus removal capacities, we conducted tests in which extract ET and three types of virus were loaded. The results were that leaks of live virus were observed in membrane modules of the same type as the type whose membrane modules permitted leaks of live virus previously and that ET began to show gel activity, which was not observed immediately after infiltration, in 24 to 48 hours. But judging from the operations of modules under ordinary conditions and conditions of contamination of raw water, we feel the possibility of leaks as described above occurring is extremely small.

The foregoing test results have shown us that the LAL test needs to be further refined and that the phenomenon of ET becoming LAL positive in 48 hours which was detected in the test of loading ET on a membrane needs to be explained immediately from the perspective of membrane evaluation as well as of preventing accidents in clinical trials, and at present we are engaged in such work. Studies need to be made on how to remove eluates from dialysis membranes or how to remove ET from dialysis modules. We consider these are grave problems for medical practice that need to be solved urgently, along with the aforementioned problems, and we plan to work to solve them in the last year of this project. A wide variety of polymer membrane materials are expected to be developed in the near future; we believe studies on their performance evaluation must be promoted simultaneously.

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Research on Biological Defense Mechanisms

4306654d Tokyo SYMPOSIUM REPORT ON GOVERNMENT & PRIVATE SECTOR JOINT R&D PROJECTS in Japanese Jan 88 pp 77-105

[Text] Article by Yoshiyuki Morishita, chief, First Food Sanitation Office, Food Sanitation Department, National Institute of Health: "Intestinal Flora and Food Sanitation" [pp 77-78]

Fermented milk products containing live bacteria of bifidobacterium or lactobacillus acidophilus have been placed on the market. These are bacteria with a strong fermentation capability that reside in the intestines. Ingestion of fermented milk products in general has the function of suppressing putrefaction in the intestines by controlling the disturbance of the intestinal flora and changing it to a fermentation type of flora.

Another approach is to change the intestinal flora into a fermentation type of flora by eating a meal containing the right combination of foods. Ingesting hard-to-digest sugars (examples: milk sugar, oligosaccharide), on the other hand, provides those sugars to the fermentation of intestinal bacteria and the result is formation of organic acids as metabolic by-products. This is the origin of fermentation in the intestines and has some common features with the aforementioned ingestion of fermented milk products.

It may be said that human beings are destined to feed their intestinal flora from birth to death. The flora carries out various kinds of metabolic activities, so trying to form an intestinal flora helpful to the promotion and maintenance of health by digesting the right foods, the basis of metabolism, is a very important factor.

From the foregoing, we shall introduce some of the results of our experiments on what effects various kinds of diets have on the intestinal flora, and further on biological reactions, which we conducted mainly using rats. Then we shall briefly consider the relationships between meals, intestinal flora and health.

We feel the urgent problem which must be addressed at present is clarification of the relationship between the early-stage formation of intestinal flora in a suckling and its living environment. Last year and

the year before last, outbreaks of infant botulism occurred in Japan. The disease is caused by *Clostridium botulinum* that infect the colon and produce toxins. Chances of the orally ingested *botulinum* (their spores) multiplying in the colon are nil in infants whose intestinal flora are at an advanced stage of formation, much less in adults. We may safely assume that the reason *botulinus bacilli* can multiply in the colon of a suckling is because the formation of its intestinal flora is not yet complete. Ironically, the delay in the formation of the initial stage flora seems to come from a superior state of hygiene in the child-rearing environment. That is, it is a culmination of various factors such as the practice of giving birth at home has changed to deliveries in hospitals and the contact between mother and child has become less intimate. We shall give some of our opinions on intestinal floras in breast-fed and formula-fed infants.

Article by Shoshichi Nojima, professor, Faculty of Pharmacy, Teikyo University: "Blood Platelet Activating Factor" [pp 79-82]

(Objective) The plate activating factor (PAF) is a molecular species of a phospholipid of a class of esters. Besides activating platelets, it triggers a variety of physiological functions, especially lowering the blood pressure level, enhancing the permeability of blood vessels, activating white blood cells, and facilitating contraction of ordinary muscles, in extremely low concentrations, so it is drawing attention as a new mediator of inflammation and allergies. In order to understand the physiological and pathological roles of PAF, a reliable system of determining PAF and its metabolic products must be established. The methods being used at present for the detection and determination of PAF in the body are mainly bioassay methods using the activation of blood platelets as the guide. While having an advantage in the form of high sensitivity, these cannot be called established methods since they have problems with respect to repeatability because the blood platelets are unstable. Therefore, aiming at development of a simple yet reliable determination method, we tried to produce an antibody to PAF. We believe that besides having applications in the detection and determination of PAF in the body, an antibody specific to PAF can be used in a variety of analyses, such as of the behavior of PAF in cells that produce PAF itself and its target cells and PAF receptor structure.

(Method) By covalently bonding PAF analog: F1 (Figure 1) with carboxyl groups to bovine serum albumin (BSA) by means of carbodiimide on the alkyl chain terminals of the nonmetabolic agonist (1-O-octadecyl-2-O-(N,N,-dimethylcarbamoyl)-sn-glycero-3-phosphocholine), we prepared a hapten carrier complex which had 6 to 7 molecules of F1 bonded per molecule of BSA. Using this hapten antigen, we tried to produce rabbit polyclonal antibody and mouse monoclonal antibody.

A mixture of the hapten antigen and Freund's total adjuvant was intravenously injected into the back and a side of a toe of a rabbit (New Zealand White species). Two weeks after the final immunity, the total blood was extracted through the carotid artery. The antiserum was formulated by means of 50 percent saturated ammonium sulfate precipitation and protein A-cephalos column chromatography and was used as an IgG

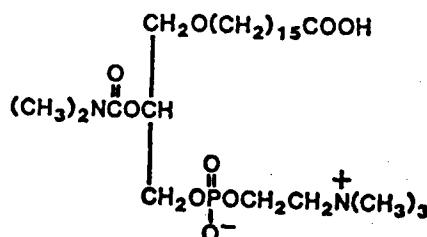


Figure 1. Structure of F1

fraction. For the production of the mouse monoclonal antibody, a mixture of the hapten antigen and Freund's total adjuvant was injected into the abdominal cavity. Three days after the final immunity, the spleen was taken out, and was fused using myeloma P3U 1 and polyethylene glycol to produce the antibody. Detection of the antibody was made by ELISA and immune precipitation. As the antigen to be adsorbed to a solid phase, we examined F1 covalently bonded to albumen albumin and PAF itself. In the case of the latter, cleaning was conducted using PBS not containing any surfactant. In the immune precipitation, the three-PAF, antibody and protein A—"sapharol"--were mixed, and it was seen that PAF does not exist in the supernatant fluid of the mixture centrifuged in a PRP coagulation experiment using rabbits. The antibody's specificity was examined in a competitive inhibition experiment of ELISA. The effect of the antibody on PAF induced drops in the blood pressure level of rats was examined by the following method. Using a male rat of the SD family that retained spontaneous breathing under sodium pentobarbital anesthesia, we measured the animal's arterial blood pressure using a transducer placed at its left femoral region. Drugs were injected via canula inserted into the rat's femoral artery. Doses were 0.2 ml/kg for PAF and 0.4 ml/kg for other drugs. Immediately after injection of the medicinal fluids, the drug residues in the canula were washed out using 0.25 ml of a physiological saline solution.

(Results and Consideration)

1. It is widely known that PAF disintegrates and is rendered inactive rapidly by acetyl hydase, an enzyme that disintegrates PAF, that exists in blood and tissue. It is considered difficult to produce an antibody by using PAF itself as the antigen. So, we used PAF analog: F1, which, because of the substitution of dimethyl carbamoyl group for acetyl group, is not subject to enzymatic metabolism, as a hapten antigen. It was disclosed in a solid phase ELISA that although this antigen did not react to albumen albumin, it strongly reacted to albumen albumin covalently bonded with F1. This indicates that the antibody has recognized hapten: F1. Next, we conducted ELISA by letting PAF adhere to a solid phase. It was discovered that preincubating 0.463 microgram of rabbit polyclonal antibody with various concentrations of PAF competitively inhibits the reaction between the PAF adsorbed to the solid phase and antibody (Figure 2).

In the standard curve, the amount of PAF necessary for 50 percent reaction inhibition is 345 pmoles. From the results of bioassays, the amount of PAF

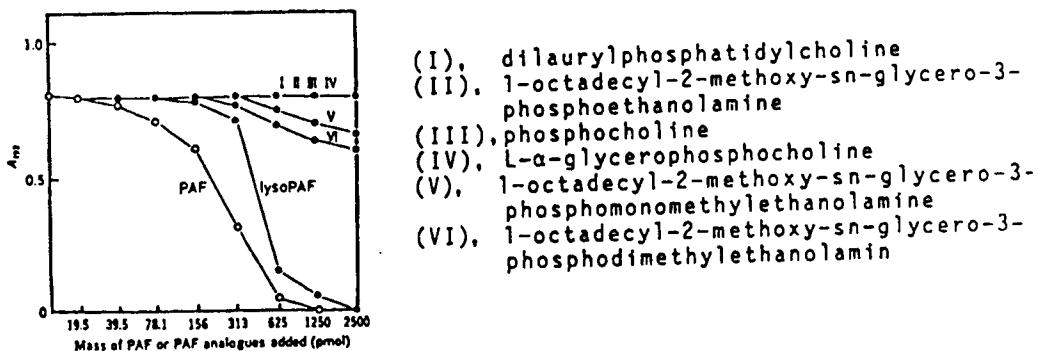


Figure 2. Specificity of PAF Antibody

in the body (for example, in a human stimulating neutrocytes, a group of 10 cells is said to produce 40 to 196 pmoles of PAF) is extremely low in concentration, so with existing measuring systems, a large number of samples are required. Thus, the measuring sensitivity must be further improved. We also examined our antibody's reactivity to PAF by immune precipitation. It was discovered that the supernatant fluid obtained by centrifuging a mixture of the three substances of PAF, antibody and protein A-cephalos does not cause PRP coagulation in rabbits.

2. Specificity of PAF antibody

Taking advantage of the competitive inhibition reaction in ELISA, we examined PAF antibody's cross reactivity. The results revealed that the antibodies, even in high concentrations, do not trigger any reaction inhibition at all with phosphocholine or glycerophosphocholine, thus proving that the PAF antibody does not cross with either of the latter two substances. The PAF also did not show any reaction to substances whose polar head choline has been replaced by ethanolamine, monomethyl-ethanolamine or dimethylethanolamine. About 50 percent of PAF antibodies crossed with the PAf receptor inhibiting drug CV-3988. Fairly large numbers of PAf antibodies cross with lysoPAF, an inactive type metabolite of PAF, so depending on the objective of the experiment, the sample to be measured may need to be isolated and purified.

We trial produced monoclonal antibodies, and the ones so far obtained were almost identical with the polyclonal antibodies in terms of potency and specificity.

3. Applications of PAF antibody

As an example of PAf antibody application, we examined the effect of PAF antibody on the blood pressure lowering function of PAF. Our results revealed that 30 minutes after administering in a mouse IgG up to 2.5 mg/kg, no inhibitory effect was observed in the PAF's blood pressure lowering function, but that 30 minutes after administering mouse anti-PAF monoclonal IgG (1.24 mg/kg), the PAF's blood pressure lowering function was completely inhibited. The PAF's blood pressure lowering function is dose

dependent (10-50 ng/kg), and it has been confirmed that the function can be completely inhibited by the anti-PAF drug, CV-6209 (50 µg/kg).

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Article by Mitsuo Honda, chief researcher, Cellular Immunity Department, National Institute of Health: "Determination of Human Interleukin-2 Inhibitor and Its Characteristics" [p 83]

The human interleukin-2 (IL-2) is a sugar protein with a molecular weight of 15,000 in the case of human beings (30,000 in the case of mice). Secreted by T cells by stimulations by mitogen and various antigens, it functions as a growth hormone-like substance for many T cell subtypes. IL-2 functions via high-affinity receptors on activated T lymphocytes. Since it has become possible to produce recombinant IL-2 by cloning IL-2 and its receptor c-DNA, IL-2 is beginning to find use in clinical applications. The importance of IL-2 in the multiplication and differentiation in vitro and in vivo T cells has come to be known not only from an immunological standpoint but also from a clinical standpoint. However, there still remains much to be learned about the control mechanism of the function and it is one of the major tasks awaiting solution. We have already determined in supernatant solutions of cells from ConA stimulating mouse spleen IL-2 inhibitors with molecular weights of 12,000 and 80,000 and revealed that they are new lymphokines produced by T cells. Furthermore, by using ConA stimulating T cells we produced T cell hybridomas, cloned a strain that produces IL-2 inhibitors, and isolated and purified IL-2 inhibitors from the supernatant solution of a culture. In this research we determined human IL-2 inhibitor and studied its features. When human peripheral blood monocytes are stimulated by various mitogens, in the case of ConA stimulation there is observed apparent IL-2 inhibitor activity (10 to 20 U/ml) in 4 to 7 days after the culture was started when the IL-2 activity begins to drop. The molecular weight divides into 10,000, 40,000, and 80,000. Therefore, using drug-resistant CEM cells we formed a T cell hybridoma. Its activity was similar to the activity seen in ConA stimulation. From the foregoing, we believe that the IL-2 inhibitor is a kind of lymphokine produced by T cells. We screened cell strains (120 strains) that produce IL-2 inhibitors and determined a cell strain that isolates 20 to 50 times more IL-2 inhibitor activity than humans. The inhibitor in its supernatant solution was similar to the inhibitor described above and it proved to be a substance partially resistant to heat. The inhibitor also partially inhibited ^{125}I -IL-2 from bonding with its high affinity IL-2 receptor. Even measurements with the high-sensitivity IL-2 receptor fluorescence ELISA method that we have developed failed to show existence of soluble IL-2 receptor activity in the mentioned inhibitor segment.

Article by Kuniaki Terado, section chief, Second Research Department, Tsukuba Research Laboratory, Eisai Co., Ltd.: "Research on Collagen Arthritis in Crab-Eating Macaque" [pp 84-85]

Chronic articular rheumatism (RA) in humans is considered to be an autoimmune disease but the mechanism of its onset is scarcely known. Because in some families of mice and rats multiple arthritis (collagen inflammation of a joint: CIA) quite similar to RA is triggered by immunizing them with Type II collagen and in the blood and on the articular cartilages of RA patients exists an antibody specific to Type II collagen, it is strongly suggested that autoimmunity to Type II collagen in humans is involved in the onset of RA.

However, it has been pointed out that there are differences between human RA and CIA in rodents probably due to differences in species. Thus to understand the mechanism that triggers the onset of RA, it is becoming important to conduct research using monkeys which are much closer to humans. Therefore, by immunizing crab-eating macaques with chicken Type II collagen (C II), we examined the following: the differences in the rate of developing arthritis between males and females; features of pathological tissue images; the form of producing an antibody to one's own Type II collagen (MK II); and the antigen determining group, arthritogenic epitopes, involved in the onset of arthritis on Type II collagen molecules.

Using wild crab-eating macaques and crab-eating macaques raised by humans 3 to 4 years old, we prepared an emulsion of Freund's total adjuvant and CII so that C II is contained at the rate of 1 mg/ml. Each animal was inoculated with 1 ml of the emulsion at 20 sites on its back skin. The results were that different from mice and rats, different sexes of crab-eating macaques developed arthritis at different levels. The onset of arthritis was observed in 10 out of 10 cases in females while the rate was 1 in 4 in males.

Arthritis developed in 4 to 10 weeks after immunization, and swelling developed on four legs symmetrically on the right and left. Inflammations lasted 1 month at the shortest and 10 months at the longest. Arthritides were not limited to small joints (DIP, PIP, MP) but were observed in the hand joints and leg joints, and furthermore in the knuckle joints and coxae in some animals. Pathological tissue image pictures up to 1 month after the onset of disease revealed typical acute inflammations, showing infiltration by neutrocytes, the formation of granulation and accompanying destruction of the joint structures.

Changes in the antibody titer in the blood were apparently different from those in the mouse. While the antibody valence to the immunized C II stayed at high levels for 6 months, the antibody titer to one's own MK II peaked in 1 to 3 months and then dropped sharply within 6 months, showing a rough correspondence to the progress of inflammation of the joints. In males who did not develop arthritis, the anti-C II antibody titer tended to be higher than that for females, but the anti-MK II antibody titer showed significantly lower values, thus indicating the involvement of a specific immune inhibition mechanism to one's own antigen.

The anti-MK II antibodies isolated from the serums of these monkeys by affinity chromatography showed roughly similar cross reactivities to Type II collagens of chicken, humans, bovines, and rats as well as mice, showing that they recognize the common antigen on hetero-Type II collagen molecules. In other words, it was inferred that the anti-MK II antibody closely resembles the anti-Type II collagen antibody found in the serums of human RA patients.

Then, using CNBr dialysis peptides of C II (CB-peptide 8-12) we examined the recognition segment on the collagen molecule of the anti-MK II antibody. Our results revealed that although each had its own recognition pattern, all the anti-Type II antibodies recognized the entire fragments of CB-8 through 12. In other words, while in the DBA/mouse arthritogenic epitopes are localized in CB-11, in monkeys arthritogenic epitopes may exist in fragments other than CB-11, showing similarity to the versatility of reactions of the anti-Type II collagen found in the serum of a human RA patient.

From the foregoing, it may be said that compared to CIA of rodents, CIA in monkeys is a better model of human RA. Consequently, in order to understand the mechanism of the onset of RA and to develop treatment methods, we should consider an effective use of this model.

Article by Takao Kosaka, chief, Immunity Research Office, Immunoallergies Research Department, Pediatrics Research Center, National Pediatrics Hospital: "Role of Complement Protein C-4 in Defense Mechanisms of the Body" [pp 86-87]

Foreword

The complement protein C-4 forms C-3 convertase via activation of the classical pathway, and it, along with B factor--the factor involved in the activation of the alternative pathway--is a key factor in complement activation. Consequently, to understand the activation of C-4 would be very helpful in understanding the mechanism of activation of the complement. As the diseases that trigger activation of the classical pathway, we selected a SLE patient serum (C3 C4 lower value) and a serum that causes cold activation (C3 normal, C4 lower value) and examined the mechanisms of their activation. Described below are interesting findings we obtained in the course of comparison studies of the two sera. To measure C4, measuring methods based on the amount of protein and activation were used, but as the indicator of activation, measurement of C4d is of great use. As a method that makes it possible to measure both C4d/C4 all at once, we developed a two-direction rocket method and examined the behavior of C4d in diseases, the results of which are also described.

The C4 protein comes in C4A and C4B. Each has a different gene position and is located in the vicinity of HLA DR. Therefore, C4 is called, along with C2 and B, class III antigen. C4 protein has a close relationship with 21-OH dehydrogenase, and its DNA arrangement has been reported. The protein has also been reported to be involved in other kidney diseases,

rheumatism, and diabetes. We studied allotypes of proteins and their genes' multiple types, and described below are the results of our research.

Method and Results

We measured C4d by a two-direction rocket method. With the method, the sample is added on to a right gel of endosmosis properties that contains antibodies to C4d and C4, and rockets of C4d and C4 are formed, the former in the anode region and the latter in the cathode region. This method makes it possible to isolate and determine C4d and C4 all at once, and further makes it possible to measure trace amounts since the rocket-like nonprecipitation lines of the two do not overlap. Using the method we measured kidney disease and Kawasaki disease, and the results revealed that in serious cases large amounts of C4d are produced despite the lack of the phenomenon of complement protein amount, revealing the activation of the complement.

As a means for undertaking the activation mechanism of CR→C3, the activation mechanisms of C4 by SLE and cold activation are interesting because both are symmetrical. In the activation period of SLE, the C4→C3 activation progresses. This time, the IgG of SLE has a function to block C4BP, protecting activation in humors and promoting activation of C3. Since such a function does not exist in sera of cold activation, we assumed C4d is produced in vitro under the effect of C4BP. Besides the finding that the conditions of "cold" in cold activation are that IgG and IgM agglutinate in a state of "cold," we have shown the mechanism of cold activation, that is, the progress of activation toward C4 under the influence of CLINH.

As for the problem of genetic multiplicity in C4, studies of genes by Eco RI revealed wide heterogeneity and they were divided into seven types. We studied patients who showed lower C4 values and clarified their genetic forms.

Conclusion

We have described that C4 is an important tool in understanding defense mechanisms of the body through measurements of break down products, and understanding activation mechanism methods, and its meaning in connection with genes.

Article by Hideaki Hagiwara, director, Hagiwara Health Science Research Institute: "Research on Human X Human Hybridoma and Monoclonal Antibody IgA" [pp 88-89]

A large variety of antibody molecule groups form the humoral immunity in the human body, but it is possible to produce on a stable basis monoclonal antibodies carrying a single idiotope and paratope outside the body using human x human hybridoma. On the other hand, it has been found that the human immune system can ultimately produce antibodies not only against immunity sources that invade the body but also against elements making up one's own body from studies of autoimmune diseases and cancer. With this basic technology and phenomenon in mind, we have been producing human

monoclonal antibodies against cancer cell-related antigens by means of cell fusion of lymphocytes from cancer patients and human B cell lymphoblasts that produce no antibody. Using fusion partners of the B cell lymphoblast line that we have so far established at our institute, we have been producing, based on a nonserum culture method, class G and M and class A antibodies from human X human hybridomas prepared from lymphocytes of stomach, breast, liver, and colon cancer patients. In particular, the IgA-producing human X human hybridoma (COLNE 10 C.3) was obtained from lymphocytes of transverse mesocolon cancer patients, and TOH/B9 obtained by fusing with lymphocytes from liver cancer patients.

As for COLNE 10C3, lymphocytes from lymphatic glands in the vicinity of a cancer lesion (2×10^7) and UC729-6(1×10^7) were fused in the presence of PEG 1500 and were cultured in a HAT selective medium for 3 weeks. Five clones were observed in 96 holes. One clone was producing IgG and another clone was producing IgM, but the remaining three were producing IgG. We selected one out of the IgM producing clones, and cloned it again to produce COLNE 10C3. In order to produce TOH/B9, we fused lymphocytes from lymphatic glands in the vicinity of a cancer lesion (1×10^7 cells) and HIM/T01 (1×10^7) in the presence of PEG 1500 and cultured them in a HATO (hypoxanthine, amethopterin, thymine, "oerbine") selective medium for 3 weeks. Out of 11 clones found in 144 holes, 13 were producing IgG, 2 were producing IgM, and 1 IgA. We again cloned the clone producing only IgA.

IgA produced from COLNE 10C3 and TOH/B9 is purified by means of affinity chromatography. The purified COLN-IgA and TOH-IgA express isotypes (α_1 , λ) and (α_1 , λ), respectively.

COLN-IgA showed strong reactions to not only colon cancer cells (CaCo₂, COLO205) but also lung cancer cells (A549, PC-10) and stomach cancer cells (HKN-74, MKN-28) but showed weak reactions to encephaloma cells (glioma U87MG) and fibroblasts (MRC-9). TOH/B9, on the other hand, showed strong reactions not only to liver cancer cells (Alexander, Chang) but also to tumor cells such as COLO205 and A549. Antigen molecules to these antibodies have yet to be determined, but in order to learn the working mechanisms of cancer cell antigens on the body's immune system, we examined their cross reactivity with intestinal infectious bacteria by means of ELISA. Compared with normal intestinal bacterial groups (for example, Coli and E. aerofaciens), COLN-IgA and TOH-IgA showed strong reactivity to pathogenic bacterial groups (for example, parahaemolyticus, V. cholerae, and B. cerus). It was further discovered that TOH-IgA by itself has an activity to cause in vitro a coagulation reaction of positive bacteria to reactions.

Using the aforementioned experiment system and human monoclonal antibody IgA, we plan to conduct studies on 1) whether immune sources of bacteria have common epitopes with tumor antigens, 2) whether the B-lymphocytes that produce tumor cell-reactive IgA were sensitized by epitopes of viruses or bacteria, and 3) how antibodies to tumor antigens are produced via the immune system, by showing the molecular properties of antibody and antigen.

Article by Junichiro Fujimoto, chief, Pathological Research Office, Pathological Research Department, Pediatrics Medical Treatment and Research Center, National Pediatrics Hospital: "Diversity of Antigen-Determining Groups for CAL LA and Meaning of Its Expression in Tissue: Analysis by New Monoclonal Antibodies" [pp 90-91]

(Objective)

At present, a large number of monoclonal antibodies (MoAb) are being used to analyze the qualitative and quantitative changes of cells responsible for immunity which defend the body. But it has come to be known that among these monoclonal antibodies are some that show different levels of reactivity to the same molecule or some that become positive to quite different cell lines. What causes such differences? To show the cause or causes at the basic level is an indispensable precondition for correctly judging the behavior of cells responsible for immunity. In this research, we conducted studies on the diversity of antigen-determining groups using Common ALL antigen (CALLA) that shows a specific distribution in blood system cells and on homology of CALLA expressed in various tissues.

(Method and Results)

1. Establishment of Anti-CALLA Antibody and Study of Its Specificity

We immunized ALL-derived cell strain KM3 and conducted a cell fusion. We conducted renal immuno-dyeing for screening and established clones IF3-7 (all are IgG1 except IF5 which is IgM) that react against only glomeruli and proximal renal tubules. We compared these clones with the anti-CALLA antibody J5 against various types of cells and tumors, and confirmed that IF3 through 7 show an identical distribution to that of J5. Furthermore, from the fact that IF3 through 7, as with J5, immune precipitate a molecular weight of 100,000 dalton of protein from the cell surface, it has been known that IF3 through 7 are antibodies that reach CALLA.

2. Studies on Diversity of Antigen-Determining Groups

Using a flow site meter we studied if the antibody CALLA could prevent biotin IF3-7 from binding to the cell surface and studied the diversity of the determining groups. The results revealed that IF3-7 reacted to the same determinant group or very similar determinant groups. Probes by IF antibodies and the established anti-CALLA antibody have shown that there are at least three different kinds of determinant groups on CALLA molecules.

3. Expression of CALLA on Tissues

CALLA has been expressed not only in immature lymphocytes but also in granulocytes and the kidney. So, we conducted a study to see if CALLA found in various cell lines was of the same molecule or not. The fact that in granulocytes and the kidney, all of the aforementioned anti-CALLA antibodies showed reactivity suggests that CALLA found in different tissues share several antigen-determining groups and that all the CALLA are of the

same molecule. In the granulocytes, however, we found a large divergence in the levels of reactivity among the antibodies. Our study using neuraminidase revealed that part of the differences in reactivity is caused by different structures of the sialic acid on the sugar chain extremities.

4. Biochemical Analysis of Homology of CALLA

CALLA of lymphocytes have a molecular weight of 100,000 but those of granulocytes have a molecular weight of 110,000. Since various anti-CALLA react to them as shown above, they have a high level of homology. To confirm this, we conducted one-dimensional peptide mapping. The result was that CALLA of granulocytes showed a completely similar mapping pattern with CALLA of lymphocytes. From this, it was confirmed that the two types of CALLA have homology at the level of peptide.

(Consideration)

CALLA are important antigens that exist in unmatured B lymphocytes, and they are considered helpful in the analysis of immune cell reactions in the body's defense mechanism. In this research, using new MoAb against the molecules we determined the diversity of antigen-determining groups on the molecules and confirmed the homology of the molecules existing in various tissues by the expression of the determinant groups and at the peptide level. Furthermore, it was shown that the phenomenon in which different reactivities are seen among anti-CALLA antibodies is affected by the sugar chain structure on the part of the antigens. Recently, many MoAb have been produced and used in pathological analyses. But, accurately determining reactions of cells responsible for immunity done for the pathological analysis of disease must be conducted on the strength of the accumulation of the basic findings as described above. We plan to consider the yet-to-be-discovered meaning of CALLA in lymphocytes.

Article by Naoji Kamata, chief, Department of Experimental Surgery, Pediatrics Treatment and Research Center, National Pediatrics Hospital: "Understanding of Immune Suppression Substances Elicited by Liver Transplants" [pp 92-93]

It has been known since the 1960's that experimentally and clinically in liver transplants there are less severe rejection reactions than in transplants of other organs. The phenomenon has not been confirmed in pigs, dogs, and humans, and thus there is no worldwide consensus on this point yet. Since the establishment by our group in 1977 of a new rat liver transplant method ("Kaff method") in Cambridge, immunological specificities of liver transplants were discovered and confirmed all at once. When orthotopic liver transplantation (OLT) operations were conducted under the condition of a fully allogenic combination between the DAs (donors) and PVGs (recipients), the livers met no rejection at all and were assimilated forever. Transplants of other organs (skin, heart, kidney, and pancreas), on the other hand, were quickly rejected (7 to 10 days later) even when the operations were conducted under similar DA-PVG combinations. Following transplant of livers from DAs, we then transplanted other organs from the same DAs. The results were that rejection of these organs was suppressed

and they were assimilated forever. This phenomenon was the antigen specificity.

We then tested rat sera provided with a tolerance to see if they have an immune suppression effect or not. In other words, after transplanting a DA heart into a PVG, we then transfused (iv) 1 ml of the aforementioned OLT serum and the DA heart was assimilated forever. This OLT serum failed to extend the assimilation of a third party heart (specific immune suppression effect). Furthermore, a normal DA liver was homogenated and transfused (ip) into a PVG, and the PVG was sensitized and the DA heart transplanted into the PVG was rejected in 4 to 5 months (second-set rejection). Under a similar system, on the other hand, the DA liver for OLT (DA-PVG) was homogenated and transfused (ip) into the PVG, and the DA heart was shortly thereafter (in 2 to 3 weeks) transplanted into the PVG. The heart only cleared the first-set rejection but is beginning to be assimilated (under observation at present). The difference between normal DA liver and OLT DA liver and the mechanism of how the latter has cleared the sensitivity are still unknown. Furthermore, the mechanism of how the OLT DA liver itself triggers an immune suppression effect is unknown, awaiting further studies.

Article by Kazuo Suzuki, chief, Human Behavioral Research Office, Environmental Hygiene Department, National Institute of Pollution Research: "Transportation of Copper in Blood Flow and Meaning of Formation of Albumin-Copper-Cysteine Complex" [pp 94-95]

Elements of the same families in the periodic table show similar chemical properties, but living organisms identify or distinguish one element from another by ingenious methods. Included in the recognition process of elements by living organisms are such steps as intake of elements through the digestive tract, their absorption, their transfer through blood flow, their distribution to organs, and their excretion. The body's defense mechanism is proof that these recognition processes are functioning properly.

We conducted research aimed at showing the mechanisms of the body recognizing elements by clarifying the transport mode of the elements essential to the body in blood flow and by clarifying the processes of how metal ions and others injected directly into the blood flow are distributed among various organs and how they are excreted. This time, we conducted research on copper, a trace metal essential to the body.

Cupric chloride was injected into the veins of rats, and the relationship between the metal's speed of dissipation from the blood flow and the speed of its distribution among tumor organs such as the liver and kidney was observed. The metal disappeared rapidly from the serum, allowing the serum to recover its normal value within 2 hours. However, the speed of the metal's distribution to the liver and kidney was slow, and thus we could not qualitatively explain where the copper that disappeared from the serum went. The amount of copper distributed among the red blood cells could not fully explain the intake and outflow of the metal, nor could the amount of the metal excreted as bile and urine explain the relationship, so we

assumed that it was temporarily captured in the whole body such as walls of peripheral blood vessels.

Next, we examined how copper in the serum was transported, and what proteins it bound to. Using a gel filtration column for high performance liquid chromatography (HPLC) believed to have least interaction with the metal, we isolated serum proteins and analyzed them by the HPLC-ZCP process that uses a multi-element simultaneous detection type luminous device (ICP) as its detector. Our results revealed that in the serum almost all copper is bound to albumin.

Albumin has 35 cysteine residual groups, but 34 of them exist as disulfide bonds and only one exists as a mercapto group. This mercapto group sometimes forms an intermolecular disulfide bond with cysteine or glutathione. In such a case, albumin is divided into two groups of mercapto albumin (MAlb) and nonmercapto albumin (NALb).

The gel filtration column for HPLC that we used this time can isolate these types of albumin with high efficiency. It has been found that when copper binds to albumin in sera, albumin-copper complexes change with time. Immediately after an injection, the albumin-copper complexes that were eluted during the same retention time as that of MAlb changed, with time, into complexes that were eluted during the retention time between MAlb and NALb. After disappearance of copper from the serum, albumin gradually returned to where the original MAlb was eluted.

The results of our experiment on the behavior of copper in blood flow suggest that although copper is bound to albumin in the serum, there occurs some change in the albumin-copper complexes. It was also discovered that only copper of the albumin-copper complexes is transferred mainly to the liver.

We examined by the HPLC-ICP method to what proteins in the liver copper is bound. In the soluble fractions almost all of the copper was bound to metallochionein. This shows that uptake of copper by the liver is dependent on the amount of synthesis of metallochionein in the liver.

Using serum and refined albumin, we examined albumin-copper complexes. Details are omitted, but it was shown that the four elements of albumin (MAlb), copper, cysteine, and calcium were involved in the changes of the albumin-copper complexes with time in the sera of rats injected with copper. It was further discovered that from a stoichiometric viewpoint, a two-fold equivalence of cysteine alone was needed.

In summing up, it was assumed that copper injected into veins is carried bound to albumin and that there are time delays between the time in which the copper disappears from the blood flow and in which it is transported to other organs. All the while the copper is kept bound to the walls of peripheral blood vessels. The uptake speed of copper by the liver is dependent on the synthesis speed of metallochionein in the liver. Furthermore, it was found that in the serum, copper is bound to albumin at a 1:1 rate but the binding takes the form of complexes involving a 2-mol

equivalence of cysteine and a 1-mol equivalence of calcium. Studies are underway on the relationship between the formation of complexes and their distribution among organs of the body, as well as on its role in the identification of elements.

Article by Kazuo Suzuki, chief, Antibiotic Materials and Organisms, Antibiotic Materials Department, National Institute of Health: "Measurement of Polymorphic Leukocyte Functions Using Activated Oligophagous Particles and Myeloperoxidase Monoclonal Antibody" [pp 96-98]

The importance of the mechanism of leukocytes to metabolize foreign objects has been pointed out in connection with the defense against infection of the body, especially in the fields of sterilization, cell injury, and antitumor activation. Understanding the mechanism of metabolizing foreign objects accompanying phagocytosis is particularly important from the perspective of clinical as well as basic applications. By taking note of lysosome enzymes that are involved in the mechanism of metabolism of foreign objects by leukocytes, we aim at conducting basic research on the metabolism of foreign objects by leukocytes and its applications to clinical use. In order to determine the mechanism of how polymorphic leukocytes metabolize foreign objects, we promoted the research from two approaches (I, II). The first was to produce a monoclonal antibody to the lysosome enzyme myeloperoxidase specific to polymorphic leukocytes (PMN). The second was to improve the fine particle we developed for the purpose of measuring activated oligophagous capacity into a tool for measuring various lysosome enzymes.

I. Research on Myeloperoxidase (MPO) (Consigned to Mitsubishi Petrochemical Co., Ltd.)

(Method) Purification of MPO: PMN fractions obtained by isolating blood from normal healthy volunteers by means of dextran and density gradient centrifuge processes were homogenized. The sedimentation of the homogenized PMN obtained by centrifuge was again homogenized in a 0.1 M potassium phosphate buffer solution of pH 7.8 containing 1 percent cetyltrimethylammonium bromide (CETAB). Its supernatant was sedimented in a 70 percent saturated ammonium sulfate, was made to pass through a DEAE-cephalous CL-6B column, was adsorbed and attached to a CM-cephalous column, and was eluted in 1-0.5 M potassium phosphate buffer solutions of linear density gradient type containing 0.02 percent CETAB (pH 7.8).

MPO was eluted in three separate peaks (type I, II, III). Each type of MPO was purified by "cephakrill" S-300 column chromatography into an individual product.

Manufacture of anti-MPO monoclonal antibody: The three types of refined MPO were given to mice for immunization and their spleen cells were extracted to be fused with mouse myeloma cells (P3U1) to form hybridomas. The selection of the MPO antibody-producing cell was based on enzyme-linked immunosorbent assay (ELISA). Screening in cloning was also based on ELISA.

Reactivity of MPO antibody: Reactivity with MPO was measured by Western blot, and the reactivity with cells was measured by blood smeared specimen and flow- "sitemetry."

(Results) Purification of MPO: MPO isolated from 5.2 liters of human blood was separated into three isotypes by CM-cephalose chromatography. This was the same as the result of an experiment by Yamada, et al., conducted using human promyelopathic leukemia HL 60 cell. We purified the MPO based on the method by Suzuki, et al., but an evaluation of the MPO by the RZ (reinheizahl) value (A430/A280), an index for MPO refinement, at the stage of extraction of PMN by homogenate revealed the product had a higher relative activity than that produced by the original method. As a result of CM-cephalose column chromatography, types I, II, and III were recovered at 7.9 percent, 32.5 percent, and 59.6 percent, respectively. Mice were immunized with the refined MPO-I, II, and III thus obtained. They were also used for screening monoclonal antibodies.

Construction of monoclonal antibodies: After selective culture, 84 MPO antibody positive colonies were obtained. After further cloning, 11 clones of stable antibody-producing cells were obtained. Ten of the 11 clones reacted similarly to all of the 3 types of MPO. The remaining one clone showed a relatively high reactivity to type III-MPO. Subclasses of these antibodies are IgG1, IgA, and IgM, and all were confirmed to react only to MPO by Western blot. In the reactivity tests using smear samples, PMN granules were stained well but lymphocytes and red blood cells were not smeared. They were also confirmed to react strongly against PMN by flow-sitemetry.

II. Research on Microbeads (Contracted to Toray Industries, Inc., Nippon Bunko Kogyo Co., Ltd.)

(Method) Substrate for lysosome enzyme β -glucuronidase: The activity of 4-(methylumbelliferyl)- β -D-glucuronide (4MUGL) whose reaction products are of free fluorescence and naphthol-AS-BI- β -D-glucuronic acid (NAS-BIGL) whose reaction products are of microbead-combined type fluorescence was measured using 4-methylumbelliferone (4MU) and naphtholAS-BI (NAS-BI) as the standard, respectively, on a 96-well fluorophotometer (LFA-096F manufactured by Nippon Bunko Kogyo).

Binding of 4MUGL to microbeads: 1) 4MUGL was mixed with 20 mg/10 ml of 0.1 N NaOH plus 10 ml of sodium periodate and was agitated, at pH 6.5, for 1 hour at room temperature. Then, the mixture was added with 20 ml of 1 percent "trace fair GN" (2 μ m, manufactured by Toray Techno Co.) and was agitated for 2 hours at room temperature. 2) For binding NAS-BIGL, 1 mg of NAS-BIGL was dissolved in 5 ml of 1 percent trace fair R8 (2 μ m) and was mixed, at pH 8.0, for 2 hours at 25°C.

(Results) We examined the stability of preserving microbeads. We also evaluated the oligophagous tests on two types of beads, one of the free fluorescence type and the other of substrate-binding fluorescence type. Olygophagously eaten by PMN, each showed luminescent strengths dependent on the PMN's active oligophagy. Seventy-five percent of 4 MU, a free

fluorescent substance, was let free outside of the cells. NAS-BIGL was hydrolyzed by β -glucuronidase and the refined bead-binding type fluorescent substance NAS-BI was found, together with the oligophagously eaten microbeads, localized inside the cells. The conventional 4MUGL-microbead is best suited for measuring the PMN's active oligophagy. However, from the perspective of preservation, it is low in stability. The NAS-BIGL microbead is capable of emitting fluorescent light in insoluble portion (inside the cell), and selective use of the two types of microbeads will help facilitate analysis of active oligophagy capacity.

(Considerations) We believe the use of the two types of fine particles for active oligophagocytosis and the MO monoclonal antibodies in measuring PMN's capacity will enable us to understand the detachment and differentiation of myeloid leukemic cells and the state of leukemia.

Article by Hiroshige Itakura, chief, Pathology and Nutrition Department, National Institute of Nutrition: "Understanding the Structure of Apolipoproteins and Their Expression" [pp 99-100]

Apolipoproteins are proteins that play an important role in controlling the metabolism of lipids. Of them all, Apo-B is a major constituent apoprotein of "chiromicron," VLDL, and LDL, and it is involved in the absorption, transfer, and uptake into the cell, of lipids. An increase in Apo-B, on the other hand, is clinically drawing attention as a guide to the advance (deterioration) of arteriosclerosis.

It was recently discovered that Apo-B comes in two types, the B-100 (550 Kd) derived from the liver and the B-48 (240 Kd) derived from the small intestine. Since the two types of Apo-B exist in the human body, lipids absorbed from outside the body and lipids synthesized in the liver have different functions. We believe that understanding under what mechanisms the two types of Apo-B are being controlled will contribute greatly to understanding the use of lipids in the body and pathological features.

Studies on Apo-B-100, including ours, have been promoted at several facilities, and cloning of mRNA and genetic DNA, as well as their structural analysis have been undertaken. As a result of Northern blot analysis of mRNA from the human liver and small intestine using two cDNA pieces, clones of the human Apo-B-100 (HBC3-3 (1.1-kb) and HB6P7), as probes, we observed, in the case of the probe HBC3-3 on the 3' side of Apo-B-100 mRNA, only a 15-kb band that is considered to correspond to B-100 mRNA in the liver and small intestine, but in the case of the probe HB6P7 on the 5' side, we observed not only the 15-kb band in the liver and small intestine but also a band at 70kb in the small intestine, which is considered to correspond to B-48 mRNA. Furthermore, as for the 7-kb mRNA that was considered to code for the B-48 observed in the human small intestine, we analyzed the structural difference between it and mRNA of B-100, and cloned its 3' side pieces.

Human small intestine mRNA was extracted by guanidine thiocyanate method, and poly (A⁺) RNA refined in an oligo (dT) cellulose column was used. Using the four pieces--PH1B4-1P1 (645706598b), PH1B4-1P3 (6599-6874b),

PH1B4-1P2 (6875-8128b), and PH1B4-1P4 (8129-8236b)--which were obtained by means of PstI digestion of H1B4's EcoRI piece pH1B4-1 as probes, we analyzed the small intestine Apo-BmRNA by Northern blot. The small intestine cDNA library was prepared by inserting ds-cDNA synthesized from poly(A⁺) RNA by Gublar-Hoffman method into λgt10.

In the human small intestine was observed 15-kb and 7-kb mRNA that code for Apo-B. Results of analyses using PstI pieces of pH1B4-1 revealed that the 7-kb mRNA band was observed on the 5' side and that the 15-kb and 70-kb bands were obtained when segments up to about 6.9-kb were probed. In other words, the 7-kb band was obtained when using pH1B4-1P3 as a probe, but the 7-kb band was not observed in the pH1B4-1P4 segment.

Using these segments as problems, we searched B-48cDNA clone in the small intestinal cDNA library and obtained the clone H1B302 that reacts to only pH1B4-1P3. The clone was subcloned into PUC and its structure was analyzed. The result revealed that it has poly (A⁺) on the 3' end, an input position similar to 6725b of B-100cDNA. It was further revealed that in this clone, C of 6616b of B-100cDNA was substituted by T. That is, it was revealed that the substitution of C by T leads the codon 2153 to transform from CAA (gln) to TAA, the terminal codon of translation. This has revealed that the apo-B48, like apo-B100, is composed of 2152 amino acid residues. This result suggests that there exist two apo-B genes, but the research so far has established existence of only one apo-B gene, so it is believed the mutation from C to T in the small intestine is caused in the course of transcription or the maturation course after transcription. This mechanism is a new concept, and we feel determining its mechanism and significance is an important task for the future.

We also conducted studies on the differences in the amount of expression of apoprotein mRNA in different organs of the body in hyperlipidemia and confirmed its specificity to organs.

Article by Akira Yamamoto, chief, Pathogenesis Department, National Cardiovascular Disease Center: "Basic Research on the Reverse Transport Mechanism of Cholesterol From Extremities to the Liver and Development of Technology for Activating the Mechanism" [pp 101-102]

Hypercholesterolemia is one of the risk factors in arteriosclerosis. While being transported via low-density lipoproteins (LDL) in the blood, cholesterol permeates through the intima of arteries and deposits itself there. The deposited cholesterol, on the other hand, is transported from cells to plasma via high density lipoproteins (HDL), where it is either disposed of by the liver in the course of uptake by either apolipoprotein A-I or E, or transported temporarily to the LDL system by the action of lipid transport proteins for uptake by the liver for disposal. The balance between the two speeds of supply and removal determine the accumulation of cholesterol in the walls of arteries, that is, the progress of gruel-like hardening. In the course of development of special treatment methods such as plasmapheresis, we have proven that removing lipoprotein (lipoprotein containing apo-B) from the LDL system can lead to remission in arteriosclerosis cases. A shortcoming of this treatment is that it

promotes cholesterol synthesis in the liver and peripheral tissues, thus canceling out a large part of the gain obtained from the LDL removal. Conversely, activating the HDL system not only promotes removal of cholesterol from peripheral tissues but also inhibits synthesis of cholesterol in the liver through an increase in the amount of cholesterol flowing into the liver. The mechanism of cholesterol's reverse transport (peripheral tissues to liver), however, has yet to be fully known. In our research we plan to promote basic research on the reverse transport system of cholesterol and to develop the technology that would activate this system by taking advantage of the plasma apolipoprotein and finally to use the technology to prevent increasing hardening of the arteries as well as for the prevention of aging.

- 1) Uptake of mutant LDL by macrophages, and the mechanism of discharging cholesterol into the medium analyzed: We observed the effect of drugs on the uptake of mutant LDL by two established cell lines of tissue cell-macrophage system cultured cells. Our results revealed that alloxan promotes accumulation of lipids, while the antioxidant agent ("probukole") greatly inhibits their accumulation. That is, this is considered to be evidence that peroxides play an important role in the uptake and accumulation of lipids by macrophages.

The discharge of cholesterol from macrophages loaded with lipids is promoted by the HDL added to the medium, but artificial granules containing Apo-A-1 can also function as receptors. Macrophages also can synthesize Apo-E, but when it is in a state of monocyte, it has little mRNA and stays inside the cell. It was observed that as the transformation into macrophages was innervated by TPA, the synthesis and secretion of Apo-E increased conspicuously.

- 2) Determination of the physical activities of the reconstituted apoprotein, especially the properties of HDL-like particle and its affinity to LDL receptor: Apoproteins A-1 and E are important as the factors contributing to reverse transport of cholesterol. When injected as aqueous solutions per se, they are rapidly trapped by the liver and processed there, so they have no effect as an activating agent of reverse transport. Therefore, we prepared artificial lipoprotein particles produced by attaching to particles of phospholipid and triglyceride these apoproteins, and conducted tests to see their binding to or interference with fibroblast receptors. In the case of Apo-E, in a free aqueous state no interference with the LDL receptor was observed but in a state where lipid particles coexisted, there was observed an inhibition dependent on the density of the Apo-E binding to the lipid particles, and the extent was 10 times as strong as that of Apo-B in terms of per protein mol density.

- 3) Production of human apoproteins (A-I, E) by recombinant DNA technology, and determination of physical and chemical properties of the recombinant (γ)-Apo-E thus obtained: Mitsubishi Chemical Industries, Ltd., has succeeded in the production of human apoproteins A-I and E based on recombinant DNA technology. We examined the physicochemical and biological properties of Apo-E of the two γ -apoproteins, and studied if they are the same as those of natural (serum-derived) Apo-E. Using serum or γ -Apo-E for

dimyristoylphosphatidylcholine (DMPC), we conducted experiments to see if there was any interference to ^{125}I -labeled binding to fibroblasts. Our results revealed that γ -apo-E has the same binding inhibition effect as the serum apo-E. This is to say that in our project which is aimed at showing the reverse transport mechanism of cholesterol and development of activation technology, we could substitute human apo-E produced by means of recombinant DNA technology for serum-derived apo-E. We are studying whether it is possible to extract cholesterol from cells (gigel-like hardening cavities).

Article by Shinro Tachibana, chief, Third Investigation Research Department, Tsukuba Research Laboratory, Eisai Co., Ltd.: "Neurite Extension Factor" [p 103]

Rehabilitation is helping patients who have suffered cerebral apoplexy or external injury to the head to recover their functions. This recovery is considered to be a result of the reconstruction of the networks by the living nerves. We assumed that the extension of neurites has something to do with this functional recovery and that underlying this phenomenon were neurites. Thus, we undertook this research project. Instead of using the established nerve cell strains for the assay systems, with a view to complementing the physiological factors we examined the first-generation culture systems of normal cells. We examined ganglion from chicken embryos, spinal nerve cells from mouse embryos, and cerebral nerves from rat embryos. As a result, we managed to prepare an isolated serum-free culture system of cerebral septal area cells from the Wistar line of rat embryos 17 days old. The septal area was dispersed using trypsin and cultured serum free in a polysine-coated 24-hole plate for 4 hours to examine the extension and activation of sprouts. For the culture blood, we used the modified L-15 culture blood medium that Hefti, et al., used in 1985, added with 25 $\mu\text{g/ml}$ of insulin, 100 $\mu\text{g/ml}$ of transferrin, 60 μm of putrescine, 20 nM of progesterone, 30 nM of selenite, and 100 $\mu\text{/ml}$ of penicillin G.

To evaluate the extension and activation of sprouts, we examined the ratio of round and bright cells with sprouts more than two times as large as the cell body to the entire cells by observing contiguous four to five visual fields in the well center area using a phase contrast microscope (x 100).

Before investigating extracts from various organs using the assay system, we examined the neurite extension and activation in NGF, laminin and various nerve peptides (about 20 kinds centered on those extracted and determined from the brain, and those whose existence is already known). Furthermore, we examined such elements as amines and lipids which may be extracted. The results were that, contrary to our expectation, there was observed no neurite extension and activation in NGF and various nerve peptides but there was observed a high level of activation in laminin and phospholipids. A close examination of the phospholipids revealed that high activation was observed in phosphatidyl inositol, phosphatidyl serine, phosphatidyl choline, and sphingomyelin.

Using this system, we are investigating neurite extension factors in extracts from various organs of the body.

Article by Shinichi Kozaka, professor, Physiology Class, Medical Department, Keio University: "On Functional Role of Astroglia in the Growth of Neurons" [p 104]

Using a system which was obtained by incubating neurons, in the first culture, on an astroglia gained by subculturing the brain from a rat embryo, we have been studying the effect of astroglia on the growth of neurons. We published a report that on astroglia, the existence of neurons and the extension of their sprouts are promoted, whereas their division is inhibited. This time, we examined to see if their hardening was caused by astroglia-derived liquid factors or if the phenomenon was expressed by contact between membranes of astroglia and neuron. We placed a cover slip on astroglia and then obtained the first-generation culture of neurons. An investigation to observe the existence of neurons, the extension of sprouts and their division revealed that the existence of neurons was promoted, whereas their division was inhibited. No effect on the extension of sprouts, however, was observed. Then, we killed astroglias by applying heat to them and went on to culture neurons directly on the dead astroglias, and the result was that, as with the case of live astroglias, there was observed the effect of promoting the extension of sprouts. From the foregoing, we concluded that the liquid factor has an important role in the life promoting effect and division inhibiting effect that astroglias have on neurons, and that the contact between membranes has an important role in the sprout extension promoting effect.

Next, instead of normal astroglias we used cells that were obtained on 18 subcultures of astroglias, or fibroblasts to see their effect on promoting extension of neurons. The cells (P18) coaggregated and showed no effect at all of promoting extension of sprouts. With the fibroblasts, neurons dispersed ideally, and there was observed some sprout extension effect, but the effect was not as great as that of astroglias. This suggests that the sprout extension effect of astroglias is a phenomenon specific to the molecules. This has also been confirmed in investigations by an electron microscope.

Article by Hideyuki Uchimura, director, National Hizen Sanatorium: "On Changes in Neurotransmitter Substances Accompanying Damage on Brain Circulatory Systems" [p 105]

The factors triggering the onset of cerebral infarction, which is the most common disease of all cerebral blood vessel injuries according to epidemiological surveys and clinical trials, are increasing age and high blood pressure. Using rats with high blood pressure (SHR), we examined the effects which high blood pressure and aging have on the cerebral circulation control. We also reported that monoamine is involved in the onset mechanism of high blood pressure in SHR at the cerebral stem nerve nucleus. Encephaloischemia can be easily generated in both cerebral spheres by the obstruction of the common carotid arteries on both sides, and they are established as an encephaloischemia model. Changes in the

neurotransmitter substances caused by encephaloischemia are considered to be deeply involved in not only acute stadium cerebral infarction but also chronic stadium cerebral infarction and cerebral blood vessel-caused dementia. We examined changes inside the brain of neurotransmitter substances, especially monoamine, by encephaloischemia, using SHR experimental encephaloischemia models.

Using 5-month old SHR, we divided them into three groups of 1) 20-minute encephaloischemia group, 2) 20-minute blood recirculation after 20-minute encephaloischemia group, and 3) sham ope group and cut out from frozen contiguous segments frontal lobes corpora striata, and hippocampi. We then measured dopamine, serotonin, and norepinephrine, and their metabolic products, using HPLC-ECD. Furthermore, using rats of group 2), we conducted in vivo brain dialysis of corpora striata and measured changes in dopamine release with time. We also examined the content of monoamine in cerebral cortices and corpora striata of two groups of SHR, one 5 months old and the other 15 to 20 months old, to see how it changed with aging.

In the cleaved brain tissues the amount of dopamine increased in both the corpora striata and frontal lobes after the blood flow was restarted, but there was no change in group 1). No appreciable changes in serotonin and norepinephrine and their metabolic products were observed. In the in vivo brain dialysis, on the other hand, extracellular dopamine in ischemia increased about 200-fold and recovered its previous value after the resumption of blood flow. The amount of DOPAC, the metabolite of dopamine, decreased to 50 percent of its original value in 20 minutes of ischemia and increased to 200 percent after the resumption of blood flow. The foregoing findings indicate that when dopamine is discharged in large quantities, out-transport of DOPAC to outside the cells is adversely affected. We plan to conduct studies on other neurotransmitter substances and the state of chronic stadium.

In old SHR the amount of monoamines and their metabolites was about the same as that of 5-month old SHR. We plan to conduct studies on changes in SHR of various ages when encephaloischemia is applied.

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Budget for Joint Projects Outlined

43066554e Tokyo SYMPOSIUM REPORT ON GOVERNMENT & PRIVATE SECTOR JOINT R&D PROJECTS in Japanese Jan 88 p 107

[Text] (Material) Outlines of Government and Private Sector Joint R&D Projects for Life Sciences in 1987

	Applications	Adoptions
Government funded	192 projects ¥720 million	173 projects ¥600 million
Private sector funded	179 projects ¥360 million	171 projects ¥310 million
Total	371 projects ¥1.08 billion	344 projects ¥910 million
1st field	Biotechnology	¥320 million
2d field	Medical materials	¥280 million
3d field	Defense of the body	¥310 million
Total	¥910 million	16 themes 48 tasks

Participants:

National research institutes: Ministry of Health and Welfare
(14 institutes)
Environmental Agency (1 institute)
Total: 15 institutes

Private enterprises: Member companies of HS Foundation (74 firms)
Nonmembership companies (21 firms)
Total: 95 firms

Universities: University of Tokyo, and others
Total 14 universities

Test and research foundations: Food and Drug Safety Center and others
Total: 14 institutes

The government-funded ¥600 million is the same as that for last year, but the private money increased by about ¥50 million over last year. This is due to the fact that the budget frameworks for consignment projects from the National Institute of Health and the National Institute of Hygienic Sciences increased a combined total of about ¥20 million and that payments for consignment projects from the National Mental and Psychiatric Center, the National Pediatrics (Children's) Hospital, and the National Rehabilitation Center for the Disabled have increased.

Adopted research tasks (including subtasks) in each of the research fields are shown in Table 1 [see page 1].

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